ENTEROPATHOGENIC ESCHERICHIA COLI (EPEC) AND OTHER PATHOGENS IN HOSPITALISED CHILDREN WITH DIARRHOEA

BY

DR. RABI BISWAS

THESIS

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PREFACE

This study for my M. Phil degree course started in February, 1994 and was initiated by Dr. Peter B. Sullivan. However as Dr. Sullivan was due to leave Hong Kong, Dr. Lewindon was assigned to be my supervisor, and with the help of Dr. Lyon of the Department of Microbiology, the design details of this study were discussed. Collaboration with Professor Asa Ljungh of Lund University in Sweden was also planned to perform the detailed analysis of *E. coli.*

The first five months of study involved undertaking a literature review and designing and piloting the questionnaire. Laboratory training for 2 weeks was also undertaken within this period. Finally in the 1st week of August, 1994 I began to recruit patients, collect specimens and process *E. coli* colonies for further analysis.

Unfortunately anticipated financial support for the study did not materialise. Thus the proposed screening of the saved *E. coli* colonies with Rosco Double-Test tablet was not possible. More concerning was the failure of the planned overseas collaboration for *E. coli* analysis because of financial constraints. It was with considerable relief and gratitude that Dr. Echeverria offered to allow me to perform the necessary gene probing at the AFRIMS laboratory in Bangkok.

During this time Dr. Lewindon also left Hong Kong in January, 1995 and Dr. David Lau took over as supervisor. Unexpectedly Dr. Lau also moved to another hospital resulting in a further change of supervisor to Dr. E. A. S. Nelson. Fortunately Dr. Nelson had had some involvement with the study from the outset having been doing a related study on the home management of diarrhoea. Data collection completed in July, and I travelled to Bangkok to undertake the gene probing during August and September, 1995.

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I wish to thank Dr. Donny J. Lyon of the Department of Microbiology in Prince of Wales Hospital for his untiring advice and discussion.

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My appreciation also goes to my wife, Jharna, my parents, other relatives and friends, especially Dr. Senok and Dr. Islam. I owe them for their continuous encouragement, support and sacrifice throughout the entire research.

CONTENTS

GLOSSARY

AA	Aggregative adherence
AE	Attaching and Effacing
AFRIMS	Armed Forces Research Institute of Medical Sciences
Ap	Ampicillin
ATP	Adenosine tri-phosphate
BFP	Bundle Forming Pilus
bfpA	Bundle forming pilus A
bp	base pair
CsCl	Cesium hydrochloride
CTD	Calf thymus derivative
CTP	Cystine tri-phosphate
DDW	Double distilled water
DNA	Deoxyribo nucleic acid
eae	*E. coli* attaching and effacing
EAEC	Enteroadherent *Escaherichia coli*
EAF	EPEC Adherence Factor
EAggEC	Enteroaggregative *Escherichia coli*
E. coli	*Escherichia coli*
EDTA	Ethylene di-ethyl tetra acetate
ELISA	Enzyme linked immunosorbent assay
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
GTP	Guanine tri-phosphate
H	Flagellar antigen
HCl	Hydrochloric acid

HEp-2	culture cell
H_2O	Water
K	Capsular antigen
KDa	Kilo Dalton
LA	Localised adherence
lb	pound
LT	Heat-labile toxin
MDa	Mega Dalton
NA	Nutrient agar
NaCl	Sodium chloride
NaOAC	Sodium acetate
NaOH	Sodium hydroxide
NT	New Territories
O	Somatic antigen
^{32}P	Radioactive Phosphorus
PEG	Poly-ethylene glycol
pH	Negative logarithm of H^+ concentration.
PWH	Prince of Wales Hospital
QMH	Queen Mary Hospital
SDS	Sodium dodecyl sulphate
SET	Sodium chloride, EDTA and Tris
SLT	Shiga-like toxin
SSC	Sodium citrate, sodium chloride
ST	Heat-stable toxin
TB	Tris-boric
TE	Tris-EDTA
TES	Tris-EDTA-sodium acetate
TTP	Thiamine tri-phosphate
VP	Voges-Proskauer
VT	Vero toxin
WHO	World Health Organisation

ABSTRACT

To determine the prevalence of Enteropathogenic *Escherichia coli* (EPEC) and other traditional enteropathogens in hospitalised children with acute diarrhoea in Hong Kong, 388 children with diarrhoea and 306 children of similar age range without diarrhoea were evaluated during one year period from August 1994 through July 1995 in Prince of Wales Hospital. Patients with a history of diarrhoeal episode or antibiotic therapy within two weeks prior to admission and those with a history of recent significant gastrointestinal disease were excluded. All stools were cultured for *Salmonella, Shigella, Vibrio, Campylobacter and Aeromonas*. Microscopy was performed for ova and cysts of diarrhoeagenic parasites. In addition, patients with diarrhoea were investigated for rotavirus by ELISA. For the identification of *Escherichia coli (E. coli),* stools were cultured on MacConkey agar and presumptive *E. coli* colonies from 253 cases and 177 controls were saved. Colonies were further analysed with eight DNA probes specific for EPEC and other 3 categories of *E. coli*. Of the diarrhoeal cases, 55% were under one year and 80% were below two years of age. On admission, 22% had some dehydration but none were severely dehydrated. Over 60% of children with diarrhoea had one or more pathogen(s) in their stool. Among the cases, rotavirus was the most commonly isolated pathogen (34.6%), followed by *Salmonella* (23.3%), *Campylobacter* (4.7%) and *Shigella* (2.1%). EAF (EPEC Adherence Factor) positive EPEC were isolated only from 10 of 388 (2.6%) cases but none from the controls ($p = 0.005$). Other categories of *E. coli* and parasites were not found to be associated with diarrhoea. Rotavirus was mainly detected in the winter months (December-February). In Hong Kong, diarrhoea in hospitalised children is most commonly caused by rotavirus and non-typhoid *Salmonella,* whereas *E. coli* and parasites are not important. Although diarrhoea is responsible for a significant morbidity in young children in Hong Kong being the second most common cause for hospital admission, it is clinically a mild disease, where children are otherwise healthy and well-nourished.

Chapter-1

INTRODUCTION

1. 1. OVERVIEW

Diarrhoea is still, today, a public health hazard throughout the world and a leading cause of childhood mortality and morbidity in developing countries.

Since *Escherichia coli* (*E. coli)* makes up a significant part of the aerobic bowel flora, it was not initially suspected that these organisms could be a cause of disease. During the first half of this century a series of epidemiological studies in Germany and England suggested that certain types of *E. coli* were associated with diarrhoea [Echeverria *et al.* 1993]. However, some categories of *E. coli* are now recognised to be one of the most common causes of diarrhoea in different parts of the world, particularly among infants and children in developing countries.

Like other bacterial pathogens, *E. coli* can undergo genetic mutation(s) and acquire new genetic material. As a result, it is possible for new strains with different antigenic properties to emerge causing severe illness in humans. It is, therefore, necessary to have a world-wide surveillance system to look for the possible emergence of new pathogens.

Enteropathogenic *Escherichia coli* (EPEC) is the first category of *E. coli* to be identified as a cause of diarrhoea. With the recent advancement of knowledge, it has been shown that EPEC is responsible for a significant number of diarrhoeal epidemics in developing nations and sporadic diarrhoea in developed countries [Edelman and Levine, 1983]. It also causes endemic diarrhoea in some countries [Moyenuddin *et al.* 1987, Lim *et al.* 1992] and has been implicated in chronic or persistent diarrhoea [Clausen and Christe, 1982, Hill *et al.* 1991]. As EPEC has been proven to be a potential diarrhoeal pathogen for significant morbidity especially in children over almost every part of the world, it is very important to have detailed knowledge about its current epidemiology in areas where diarrhoea is a well recognised problem for humans.

Childhood diarrhoea is a significant health problem in Hong Kong. It is the second most common (15.8%) cause of paediatric admission in Prince of Wales Hospital [Departmental data-1993]. Unfortunately, there is little information on the distribution and incidence of enteric pathogens in Hong Kong, especially that related to various categories of *E. coli* including EPEC.

The pathogenesis of EPEC is uncertain and serotyping still remains the most commonly used diagnostic test for EPEC. Full serotyping, which defines standard EPEC serotypes [Levine and Edelman, 1984], is both time consuming and expensive and most diagnostic laboratories rely on O antigen EPEC serogrouping. But O serogrouping alone has been shown to be less reliable diagnostic test for EPEC due to its high false-positive and false-negative results [Farmer *et al.* 1977]. Furthermore, classical EPEC can be defined more precisely on the basis of unique genetic determinants that encode characteristic pathogenic properties. To determine the prevalence of EPEC in hospitalised children with diarrhoea Hong Kong, a prospective case-control study was conducted over a one year period from August, 1994 through July, 1995 in Prince of Wales Hospital. Assistance of a research laboratory in Bangkok was given where there are the latest facilities for gene probing.

1.2. OBJECTIVES OF THE STUDY

The objectives of the study were as follow:

- To determine the prevalence of EPEC in hospitalised children with diarrhoea in Hong Kong.
- To determine the role of other common enteropathogens responsible for childhood diarrhoea.
- To categorise *Escherichia coli* strains identified.
- To identify specific virulence factors in the *E. coli* strains isolated.
- To correlate the clinical features with the aetiologic pathogens.

Chapter-2

LITERATURE REVIEW

2.1. BACKGROUND OF THE STUDY

Diarrhoea is one of the most important causes of morbidity and mortality in children in developing countries and also a major health problem in industrialised countries [Lerman *et al.* 1994]. More than a billion episodes of diarrhoea are estimated to occur each year in children under-five in the developing world. In 1992, diarrhoea was estimated to be the second leading cause of death in children world-wide accounting for 2.9 million child deaths [Grant, 1994]. In addition to causing high rates of mortality and morbidity, diarrhoeal diseases are one of the main causes of childhood malnutrition.

In a retrospective analysis of the aetiology of diarrhoea in Hong Kong, no pathogens were isolated from the majority cases by routine microbiological techniques (*Salmonella, Shigella, Vibrio, Campylobacter, Aeromonas, Plesiomonas,* and rotavirus) [Ling and Cheng, 1993]. However, several micro-organisms, including *E. coli*, rotavirus, *Salmonella, Shigella, Campylobacter, Cryptosporidium* and *Giardia lamblia* have been considered as the causes of diarrhoea among the children of developing world [Branski, 1984], where *E. coli* accounts for 30% of acute gastroenteritis in some places [Moyenuddin *et al.* 1987]. Among the 5 well recognised categories of *E. coli,* several epidemiological studies have incriminated enteropathogenic *E. coli* (EPEC) as the most common cause of childhood diarrhoea in some parts of the world [Katouli *et al.* 1990, Gomes *et al.* 1991].

Like other parts of the world, acute diarrhoea in children is a leading cause of morbidity and to some extent mortality in Hong Kong [Leung *et al.* 1987]. From November, 1984 to June, 1992 there were 88 paediatric deaths recorded in Prince of Wales Hospital. This hospital is a large acute regional and teaching hospital situated in the New Territories of Hong Kong. It contains 1,342 in-patient beds including 194 for paediatric admissions with specialist out-patient clinic and emergency service facilities.

Seven of these 88 deaths (8%) were due to diarrhoea. Furthermore, diarrhoea is responsible for an average of approximately 80 monthly admissions to Prince of Wales Hospital. Of these admissions, an organism is isolated in less than 30% of cases, where tests for the isolation of pathogenic *E. coli* are not performed routinely (Source: Departmental Audit Programme (1993), Department of Paediatrics, PWH). It is possible that a significant portion of these 'organism unknown' cases are caused by EPEC.

Although complete reports on microbiology of diarrhoeal diseases in Hong Kong are not available, it has been demonstrated that *E. coli* may have a significant role in the pathogenesis of childhood diarrhoea in Hong Kong. Most of the previous works were undertaken before 1985. More recently, Ling and Cheng (1993) retrospectively analysed the data on all pathogens isolated from faecal specimens of diarrhoeal patients admitted to Prince of Wales Hospital from 1984 to 1990. Although in this study, testing for pathogenic *E. coli* was not performed unless 'clinically indicated', still several isolates of *E. coli* were obtained among the diarrhoeal patients. Association of these isolates with diarrhoea were not defined specifically.

A study performed between 1982 and 1985 in Queen Mary Hospital of the University of Hong Kong, demonstrated that 25.1% of childhood diarrhoea were caused by bacterial pathogens, e.g. *Salmonella*, *Shigella*, *C. jejuni* and *E. coli*. Only 442 of a total 2,228 samples were tested for EPEC, of which 57 (12.9%) were positive [Lam *et al.* 1989].

Another study conducted in Queen Marry Hospital by Yam *et al* (1987) during 1982 to 1985 revealed that *E. coli* was associated with 23% of childhood diarrhoeal episodes in Hong Kong, of which 2.9% were confirmed to be of EPEC serogroups. Enterotoxigenic *E. coli* (ETEC) was diagnosed by laboratory methods in 0.9% cases. Enteroinvasive *E. coli* (EIEC) and enterohaemorrhagic *E. coli* (EHEC) strains were considered unlikely on clinical grounds. In the other 19% cases *E. coli* was isolated as a predominant or pure growth on repeated stool culture and no other conventional pathogens were isolated by the available methods. Yam *et al.* concluded that the large preponderance of *E. coli* isolates were associated with childhood diarrhoea, but possessed none of the commonly described pathogenic attributes. Thus highlighting the need for

further research on the pathogenesis of childhood diarrhoea associated with these organisms.

The taxonomy of *E. coli* is becoming more complex day by day due to discovery of new virulence factors. Furthermore, due to its time consumption and expenses, most laboratories perform O antigen EPEC serogrouping, rather than full O/H serotyping [Knutton *et al.* 1989b]. But O serogrouping alone causes overestimation and thus has got less diagnostic value for EPEC [Farmer *et al.* 1977], which is being replaced gradually by molecular and other techniques. It may be due to the fact that classical EPEC serogroup may or may not contain virulence factor, or because of the existence of characteristics of different categories of *E. coli* within a single designated EPEC 'O' serogroup. For example, among the EPEC O126 serogroup, both enterotoxin production and enteroaggregation have been found [Yam *et al.* 1994]. With the advancement of molecular biology, it is possible to define classical EPEC serogroups more precisely by the DNA probes.

Since 1985, no further studies have been conducted to elucidate the specific role of EPEC in childhood diarrhoea in Hong Kong, despite the fact that enteric infection remains a major health problem in these children. The previous studies also showed evidence that *E. coli* and more precisely EPEC are possibly important in childhood diarrhoea. The present case-control study will help to clarify the role of EPEC in childhood diarrhoea in Hong Kong.

2.2. ESCHERICHIA COLI : OVERVIEW

Escherichia coli has been referred to as colon bacillus because it makes up a significant part of the aerobic bowel flora and initially it was thought that it did not cause any disease in humans. But over time *E. coli* has been the object of more scientific research. It is also one of the most commonly isolated human pathogens causing urinary tract infections [Collee *et al.* 1989] and it also causes wound infections, pneumonia, meningitis and septicaemia [Koneman *et al.* 1992]. The recent developments in the study of diarrhoeal aetiology demonstrated that in the absence of known enteropathogens, *E. coli* must be considered in the diagnosis. However, the routine hospital laboratory does not have the techniques for detecting many of these organisms. Most of the published reports

implicating *E. coli* in diarrhoeal disease are from investigations of outbreaks by public health groups or from designed prospective studies by teams of specialised investigators.

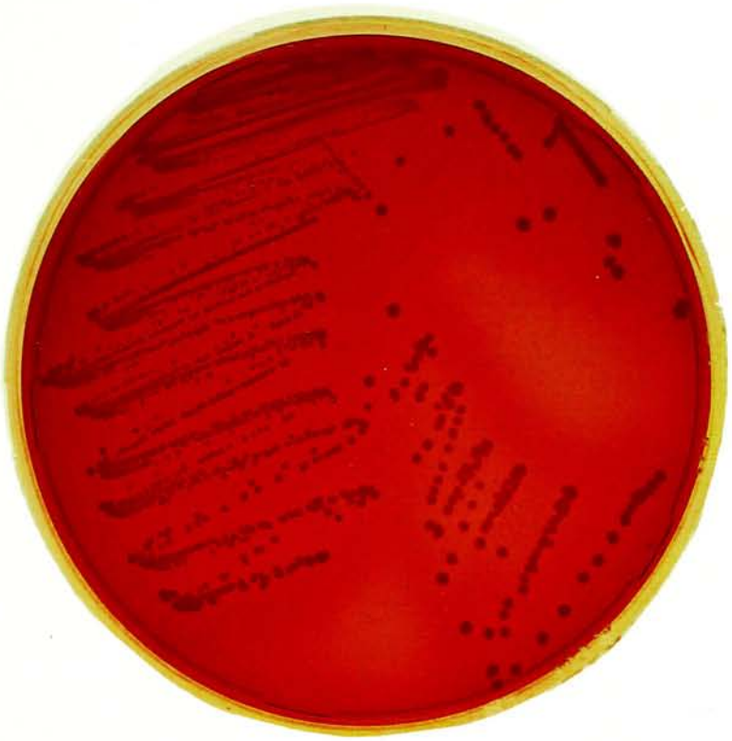
2.2.1. Morphology

E. coli is a gram-negative non-sporing bacillus. 80% strains are motile and fimbriate. A few strains are capsulate [Collee *et al.* 1989]. *E. coli* resembles other coliform bacteria both morphologically and physiologically and coliform bacteria frequently differ from one another by only minor characteristics [Carpenter, 1972].

2.2.2. Cultural characteristics

E. coli grows readily on all ordinary media and is aerobic and facultatively anaerobic [Gillies, 1984]. Typical *E. coli* colonies are recognised by their characteristic appearance on certain media. Although some strains may ferment lactose slowly, they usually appear as lactose fermenting colonies on MacConkey agar. On this medium colonies are smooth, glossy, translucent and rose-pink in colour [Collee *et al.* 1989]. Colonies are 2-4 mm in diameter and convex with an entire edge after 18-24 hours incubation at 37^0 C under O_2 [Gillies, 1984] (See Figure- 2.1).

Figure- 2.1 Morphology of* E. coli *colonies onto the MacConkey agar medium



2.2.3. Biochemical reactions

E. coli is a strong lactose fermenting coliform [Baron, 1991]. It is distinguished from other enterobacteria and defined as a species on the basis of its biochemical reaction with certain substrates. It's pattern of reactions include indole positive, methyl-red positive, Voges-Proskauer (VP) negative & citrate utilisation negative [Collee *et al.* 1989]. Like all other enterobacteriaceae, *E. coli* produces acid but unlike them produces gas from glucose and other carbohydrates [Braude *et al.* 1986].

2.2.4. Antigenic Structure

Three kinds of surface antigens are utilised in agglutination tests for the serotyping of *E. coli*: the O (Somatic), K (Capsular) and H (Flagellar) antigens. Each 'O' serogroup of

strains is defined by the presence of a different O antigen. Initially 25 O serogroups were described by Kauffmann in 1947, but over 160 such groups have been described by Edward and Ewing in 1972. Within each serogroup are one or more serotypes that are based on the H and K antigens. At least 100 different K antigens and over 50 H antigens are described today. The serotype of a strain is defined by its full antigenic formula, i.e. its O, K and H antigens, e.g. O55:K59:H6. The K antigen present only in capsulate forms includes three kinds of antigens; L, A and B, that lie outside the cell wall [Collee *et al.* 1989].

2.2.5. Identification

E. coli can be identified grossly with the help of a few limited tests, i.e. morphologically with pink, non-mucoid colonies on MacConkey agar, biochemically with fermentation of glucose and lactose, indole production and failure to utilise citrate and confirmation of motile characteristics [Collee *et al.* 1989].

2.2.6. Classification of *E. coli*

Strains of *E. coli* differ in their pathogenic potential and those of certain serotypes have a special potential for causing particular types of infection, e.g. in the intestine, where most serotypes are merely commensal. *E. coli* strains predominate among the aerobic commensal bacteria present in healthy gut. The intestinal commensal strains of *E. coli* commonly cause opportunistic infection in other parts of the body. Strains of certain *E. coli* have a primary pathogenicity in the intestine and cause gastroenteritis [Collee *et al.* 1989].

E. coli strains that cause human diarrhoea can be distinguished on the basis of distinct epidemiology, clinical syndromes and virulence properties and are classified into at least five major categories:

- Enterotoxigenic *E. coli* (ETEC)
- Enteropathogenic *E. coli* (EPEC)
- Enteroinvasive *E. coli* (EIEC)
- Enterohaemorrhagic *E. coli* (EHEC) and
- Enteroaggregative *E. coli* (EAggEC).

It has become apparent that each category of *E. coli* causes different types of diarrhoeal diseases [Donnenberg and Kaper, 1992].

The first category of diarrhoeagenic *E. coli* with definitive pathogenicity to be elucidated was ETEC, which causes small intestinal secretory diarrhoea through enterotoxin production. It is one of the most common causes of diarrhoea in young children in tropical developing countries [Guerrant *et al.* 1983], and one of the leading causes of diarrhoea in travellers [Mattila *et al.* 1992]. Diagnosis of this category mainly depends on the enterotoxin (Heat-labile toxin, LT or Heat-stable toxin, ST) liberated by it.

EIEC, which invade and multiply within the mucosa of the large bowel and produce dysentery similar to *Shigella*. EIEC and *Shigella* both produce keratoconjunctivitis when inoculated into the conjunctivae of guinea-pigs. This test is called the 'Sereny test'. There is also a specific gene-probe to diagnose this category.

The remaining strains of *E. coli* were denoted as EPEC in 1960s and early 1970s. However, the term EPEC should now be reserved for those strains that produce characteristic ultrastructural lesions on the small intestinal mucosal brush border, termed attaching and effacement (AE) lesions.

More recently, two further classes of diarrhoeagenic *E. coli* have been described; these are the EHEC and EAggEC, both of which produce an inflammatory diarrhoea [Hart *et al.* 1993]. EHEC does so by producing cytotoxin and EAggEC by its specific aggregative adherence to bowel wall. EHEC can be diagnosed by detection of high levels of cytotoxin liberated, which is active on vero cells, called Vero-toxin (VT) or Shiga-like toxin (SLT). Again there is now specific gene probe for this category. EAggEC constitute the most recently described category of diarrhoeagenic *E. coli*. EAggEC exhibit mannose-resistant adherence to cultured human epithelial cells, whereby bacteria adhere to epithelial cells in a aggregative pattern [Nataro *et al.* 1987]. Tissue culture adherence assays have served as the standard diagnostic method for the detection of EAggEC, but results may vary with variation of methodology and individual interpretation [Vial *et al.* 1990]. DNA probes which correlate with AA (Aggregative adherence) pattern are also used for its diagnosis.

Although not true for every category, there is a tendency within each category for the strains to fall into certain O:H serotypes. For example, WHO definition of EPEC

includes the following serogroups- O18, O26, O44, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 [WHO, 1987]. Even today, most laboratories depend on these serogrouping methods for the identification of EPEC.

2.3. HISTORY OF EPEC

The history of EPEC began with the history of *E. coli* more than 100 years ago. German paediatrician Theodore Escherich marked the year 1885 by the first description of *E. coli.* He demonstrated the pathogenicity of his so-called '*Bacterium coli commune*' in rabbits, not in human.

2.3.1. *E. coli* as a cause of diarrhoea

In 1889, Laurelle first suggested that *E. coli* could be pathogenic to the human intestine and these organisms caused peritonitis after intestinal perforation. He also alluded to the possibility that they caused diarrhoea and vomiting ('*cholera nostras*') [Robins-Browne, 1987]. Topley and Wilson (1936) described that at the same time, epidemiological investigation in Great Britain discovered the probable infective origin of infantile enteritis. They found a relation of epidemic diarrhoea to warm weather and termed it 'summer diarrhoea'. In fact Benjamin Rush, an American physician in 1789 had already described the first medical account of the summer epidemics of childhood diarrhoea reported in London during the second half of the 18th century. In 1897, Lessage suggested that there were both harmful and harmless strains of *E. coli* and the two could be differentiated on serological grounds. Between 1908 and 1910, Bhar investigated 117 Danish infants with diarrhoea and concluded that *E. coli* played a role in this disease. Studies conducted between 1900 and 1910 in Britain highlighted the prevalence of summer diarrhoea particularly in the first two years of life [Robins-Browne, 1987]. During the 1920s in Germany, Adam discovered a biochemically distinct group of *E. coli* which he regarded as a cause of diarrhoea [Echeverria *et al.* 1993] and named these bacteria 'dyspepsiekoli'. As an extension of Adam's work, Goldschmidt provided the serological identification of 'dyspepsiekoli' in 1930s. In 1935, Dulaney and Michelson discovered a specific colonial variant of *E. coli* , termed *'Bacterium coli mutabile'* from an outbreak of neonatal gastroenteritis [Robin-Browne, 1987].

Despite the accumulated evidence, however, general acceptance of *E. coli* as a cause of diarrhoea in humans was delayed until 1945, when John Bray's landmark article on the association of antigenically homogenous *E. coli* with 'summer diarrhoea' was published. The early epidemiological investigations received an important boost from the work of Kauffmann, who in the 1940s was perfecting a serotyping scheme for *E. coli* that was based on the bacterial antigens. In 1950, Kauffmann and Dupont employed the newly developed typing scheme to show that *E. coli* strains associated with infantile diarrhoea in different centres, including some of Adam's original 'dyspepsiekoli', belonged to serogroups O55 and O111 [Echeverria *et al.* 1993].

2.3.2. The first use of the term EPEC

Neter *et al* in 1955 demonstrated that two epidemics of nosocomial neonatal enteritis that had taken place in New York during 1947 were associated with *E. coli* O111. Subsequently experiments with *E. coli* strains of serogroups O55 and O127 showed that these bacteria, too, were pathogenic for humans. By contrast, isolates of *E. coli* from healthy individuals failed to produce symptoms when fed to control subjects. In 1955, the term EPEC was proposed by Neter *et al* to indicate those strains of *E. coli* that had been epidemiologically linked with childhood diarrhoea to distinguish them from those strains of *E. coli* that caused urinary and other extra-intestinal infections [Robins-Browne, 1987].

In 1960s the discovery of enterotoxin production by *E. coli* and the characterisation of enterotoxigenic strains of *E. coli* and their enterotoxins led to considerable confusion regarding the pathogenesis of *E. coli* diarrhoea and the nomenclature of diarrhoeagenic strains. Since many workers believed that the toxins produced by ETEC, both LT (Heat-labile toxin) and ST (Heat-stable toxin), represented the virulence determinants of EPEC, they used the terms EPEC and ETEC interchangeably [Drachman, 1974]. However, it soon became clear, that EPEC and ETEC represent distinct entities, insofar as (1) ETEC strains generally do not belong to recognised EPEC serogroups [Rowe, 1979; Evans *et al.* 1983; Gross, 1983] and (2) EPEC strains, as defined by serogrouping, generally do not produce LT or ST [Gross *et al.* 1976; Robins-Browne *et al.* 1982; Long-Krug *et al.* 1984]. These findings raised doubts about the pathogenicity of EPEC in the minds of some workers. These doubts were resolved by Levine *et al.*(1978), who successfully infected volunteers per orally and

reproduced the disease with the EPEC strains that had been implicated several years earlier in outbreaks of infantile gastro-enteritis in the United Kingdom [Gross *et al.* 1976]. Further studies with DNA (Deoxyribo nucleic acid) probes have shown that EPEC strains lack the genetic information for LT and ST [Robins-Browne *et al.* 1982].

2.3.3. EPEC as a separate category of *E. coli*

For a long time, EPEC were supposed to be identified by O serogrouping alone because no virulence factors were known for these bacteria until 1979. At that time, Cravioto *et al* (1979) reported that 80% of 51 *E. coli* strains belonging to traditional EPEC serotypes adhered to HEp-2 cells (culture cell). By contrast, only 19% of 62 *E. coli* strains of pathogenic serotypes other than EPEC were adherent. Baldini *et al.* (1983) showed that all but one of 32 EPEC strains carried 50-70 MD (mega dalton) plasmids and when one of these strains was 'cured' of a 55 MD plasmid, it lost its ability to be adherent to HEp-2 cells. Furthermore, transfer of the plasmid to a strain of non-adherent *E. coli* K-12 enhanced the adhesive ability of the recipient for tissue culture. This plasmid mediating the adherence of EPEC to culture cells was named as EPEC adherence factor (EAF) and a DNA probe was constructed from this plasmid, called EAF probe [Nataro *et al.* 1985b]. Clausen and Christe (1982) found that EPEC strains of serogroups O111 and O119 produced a distinctive pattern of adherence to HEp-2 cells that was characterised by bacteria binding in microcolonies or in aggregates, known as 'localised adherence'(LA). Since then HEp-2 cell adhesion assay and EAF probing have been used for identification of EPEC. Further detailed examinations of EPEC serogroups have shown that EPEC are a distinct category of enteric pathogens that cause diarrhoea by producing a characteristic microscopic lesion on the brush border of enterocytes [Polotsky *et al.* 1977] and which are associated with an identifiable chromosomal locus that encodes to this capability, termed *eae* (*E. coli* attaching and effacing) [Jerse *et al.* 1991]. Robin-Browne *et al* (1993) has now shown that most of the early isolates of *E. coli*, which were identified prior to 1960 and were designated as enteropathogenic were also, in fact, EPEC as currently defined.

2.4. PATHOGENESIS OF EPEC

The term *'enteropathogenic E. coli'* was coined by Neter *et al* in 1955 before the mechanisms of *E. coli* diarrhoea was understood and traditionally belonged to certain specific serogroups epidemiologically linked with childhood diarrhoeal outbreaks [Robins-Browne, 1987].

Subsequently, with the discovery of ETEC and EIEC, it was shown that the EPEC strains isolated from different outbreaks of infantile diarrhoea possessed none of the virulence determinants of ETEC or EIEC. At that time it was proposed by some authors that either these EPEC strains caused diarrhoea by some unexplained mechanisms or they shared some common surface antigens with the other strains responsible for epidemic diarrhoea [Echeverria *et al.* 1976]. It was also mentioned that these EPEC strains did not possess plasmids necessary for pathogenicity or that they might have lost the plasmid during storage prior to being studied. However, Levine *et al* (1978) performed a study to determine whether these EPEC strains might cause diarrhoea in adult volunteers. In this study, three EPEC strains isolated from different neonatal infantile epidemics and one strain from 'normal' colonic flora of a healthy adult were fed to the volunteers. This study firmly supported the contention that certain virulent EPEC strains caused diarrhoea by a mechanism which did not depend on LT or ST enterotoxin production or on invasiveness. Later, it was demonstrated that EPEC produce diarrhoea by producing an striking ultrastructural lesion on the brush border of the enterocytes [Polotsky *et al.* 1977].

Although knowledge in understanding the pathogenesis of EPEC is incomplete, the pathogenic mechanism of EPEC may be better described in relation to some specific characters. These are- (1) Plasmid encoded virulence properties. (2) Characteristic interaction with intestinal mucosa, and (3) Production of toxins.

2.4.1. Plasmid encoded virulence properties

Cravioto *et al.* (1979) observed that unlike typical *E. coli* strains, 80% of EPEC strains adhered to HEp-2 cells in tissue culture. This property was later found to be encoded on a 55 to 70-MDa plasmid, referred to as EAF (EPEC Adherence Factor) plasmid [Baldini *et al.* 1983]. Diarrhoeagenic *E. coli* can adhere to tissue culture cells in three distinct patterns: localised, diffuse and aggregative. EPEC are associated with localised adherence

pattern, which is characterised by adherence in tight clusters or microcolonies [Nataro *et al.* 1987]. This character of EPEC, to be adherent in a localised pattern to HEp-2 cells, is determined by an 'Adhesion Assay' with the help of light microscopy. It has been considered as one of the diagnostic methods for the EPEC strains. A 1-kbp DNA probe derived from the EAF plasmid of EPEC E2348/69 (O127:H6) has been used extensively in epidemiologic studies to identify and characterise EPEC strains that show localised adherence [Nataro *et al.* 1985a]. Subsequently, it was proved that EAF plasmid is necessary for the full expression of pathogenicity in EPEC that exhibits HEp-2 cell adhesiveness. It was demonstrated that diarrhoea occurred in 9 of 10 volunteers who ingested the EPEC strains carrying EAF plasmid but in only 2 of 9 volunteers who took the plasmid minus variant. The plasmid minus variant was not adherent to HEp-2 cell [Levine *et al.* 1985].

2.4.2. Characteristic interaction with intestinal mucosa

EPEC strains produce a distinctive histopathologic lesion in the human intestine, visible by electron microscopy, which cause destruction of the microvilli of the brush-border of enterocytes [Polotsky *et al.* 1977].

Recently, understanding of the interaction of EPEC with the small bowel mucosa has progressed greatly and led to a three-stage model [Donnenberg and Kaper, 1992]. According to this model, the initial adherence of the organism to the epithelial cell takes place characteristically by forming microcolonies on the cell-surface. It is recognised in tissue culture by light microscopy as localised adherence [Scaletsky *et al.* 1984] and is dependent on a large plasmid, common to EPEC strains [Baldini *et al.* 1983, Nataro *et al.* 1985b]. When the EPEC strain is attached to HEp-2 cells, it expresses rope-like bundles of filaments and rod-like fimbriae [Giron *et al.* 1993a]. Rope-like bundles of filaments are called bundle forming pilus (BFP). These together create a network of fibres which bind the individual organisms and bacteria to the eukaryotic cell membrane in a colonial mode of growth [Giron *et al.* 1991]. This colonial mode of growth seems to be an essential aspect of their pathogenicity and the cloned structural gene (*bfpA*) encoding for the BFP is highly characteristic of EPEC [Giron *et al.* 1993b].

Following the initial contact, the bacterium transduces a signal to the epithelial cells that results in the activation of host cell tyrosine kinase activity [Rosenshine *et al.*

1992]. It results in the elevation of intracellular Ca^{+2} concentration [Baldwin *et al.* 1991] and inositol triphosphate [Dytoc *et al.* 1994]. This increase in free calcium level is due to release of Ca^{+2} by inositol 1,4,5-triphosphate from intracellular stores [Baldwin *et al.* 1991]. The elevated Ca^{+2} concentration causes microvilli to vesiculate as actin is cleaved within the microvillous core [Matsudaira *et al.* 1982]. Intimin, the product of the *eaeA* gene is a 94 KDa outer membrane protein, recently isolated from sera of convalescent volunteers after experimental EPEC infection, then allows the bacteria to become intimately attached to the epithelial cell membrane [Jerse *et al.* 1991a]. Damage to the host cell microvilli filaments and a rearrangement of cytoskeletal elements follows with a proliferation of filamentous actin beneath the areas of intimate bacterial attachment [Finlay *et al.* 1992; Knutton *et al.* 1989; Manjarrez-Hernandez *et al.* 1991]. In addition to the actin, myosin [Manjarrez-Hernandez *et al.* 1992], alpha-actinin, talin and ezrin have also been identified beneath the bacterial attachment [Finlay *et al.* 1992]. Then a cup like pedestal is formed upon which the bacteria rest [Donnenberg and Kaper, 1992]. This effect has been termed attaching and effacing (AE) [Moon *et al.* 1983] and it is characteristic of EPEC. This effect is mediated by the *eaeA* gene product [Donnenberg and Kaper, 1991]. Another gene product (33-39 KDa protein) has also been identified as contributing to the intimate attachment of EPEC to epithelial cells and is encoded by the locus *eaeB,* which is located immediately downstream of the *eaeA* locus [Donnenberg *et al.* 1993b].

Invasion: There is a controversy in the literature regarding the invasiveness of EPEC. They had been considered to be non-invasive for a long time [Levine *et al.* 1978, Levine, 1987], principally because they do not cause keratoconjunctivitis in guinea pigs (Sereny test) like EIEC [Goldschmidt *et al.* 1976], *Shigella* [Levine, 1987] or *Yersinia* organisms [Levine *et al.* 1978]. However, simply lacking of this property does not preclude them for being invasive, because other invasive organisms, like *Salmonella* [Formal *et al.* 1983] and some *Shigella* strains [Sasakawa *et al.* 1986] able to invade epithelial cells are also Sereny test negative. A workshop on EPEC in 1983 declared that EPEC is not related to *Shigella*-like invasiveness [Edelman and Levine, 1983] and Goldschmidt *et al* (1976) have never seen classical EPEC to be invasive in their study on the basis of Sereny test.

On the contrary, Donnenberg *et al.* (1989) have emphasised with the results of their study that epithelial cell invasion is an overlooked property of EPEC. In favour of their opinion, although extremely rare, there is report of sepsis and death resulting from EPEC infection. Many published electron micrographs, including clinical specimens, appear to demonstrate intracellular bacteria [Andrade *et al.* 1989; Miliotis *et al.* 1989; Francis *et al.* 1991; Foubister *et al.* 1994; Ulshen and Rollo, 1980].

Recently, several investigators have challenged the traditional notion of non-invasiveness publishing descriptive [Andrade *et al.* 1989; Militois *et al.* 1989] and quantitative [Donnenberg *et al.* 1989; Francis *et al.* 1991] studies demonstrating that EPEC are capable of efficient invasion of a variety of epithelial cell types. EPEC invasion in vitro can be inhibited by cytochalasins that block host-cell microfilaments. In contrast, like attaching and effacing activity, invasion can be augmented by the presence of the EAF plasmid [Donnenberg *et al.* 1989; Francis *et al.* 1991]. The clinical significance of the invasive property of EPEC is not yet clear. However, the contribution of epithelial cell invasion to the pathogenesis of EPEC infections also deserves further investigation.

2.4.3. Production of toxins

No firm conclusion can be drawn about the production of toxins by the EPEC strains. According to the definition by the workshop committee on EPEC in 1983, classical EPEC do not produce heat-stable toxin (ST) or heat-labile toxin (LT). As knowledge has increased in the recent years about the pathogenic mechanisms of enteritis, so has scepticism about the pathogenicity of those EPEC strains, which are being identified only on the basis of the serogrouping method [Edelman and Levine, 1983]. Furthermore, it is very difficult to cause diarrhoea by the impressively short incubation period (3 to 11 hours) seen in experimental human EPEC infection [Levine *et al.* 1978; Donnenberg *et al.* 1993a] only by the loss of brush border microvilli and its consequences. As there is no evidence that EPEC affects cyclic nucleotide levels [Long-Krug *et al.* 1984], it may produces some enterotoxins other than ST or LT [Levine *et al.* 1978], which is similar, if not identical to Shiga-toxin [Edelman and Levine, 1983; Cleary *et al.* 1985]. There is a wide variation of opinions in this regard ranging from the postulation of no production of conventional enterotoxins by classical EPEC [Robins-Browne *et al.* 1982] to production of cytotoxin in low concentration by O114:NM [Bower *et al.* 1989] and overt toxin production by some

EPEC strains [Miliotis *et al.* 1989]. The continued reference to serogrouping as the causes of identifying EPEC by some workers has lead to confusion in the literature regarding the ability of EPEC to produce toxins. As stated above, EPEC is defined by the characteristic lesion on the intestinal surface, yet to have toxin production demonstrated. However, within some serogroups once classified as EPEC, there are some serotypes that do produce toxins. For example, two serotypes of EPEC O26 (H30 and H19) have been reported to produce cytotoxin active on vero cells [Marques *et al.* 1986]. The many other serotypes of O26 do not produce toxins. It is likely that these different serotypes of O26 serogroup are members of different *E. coli* categories. Indeed many serotypes have been recategorised, e.g. O26:H11 is now an enterohaemorrhagic *E. coli* [Levine, 1987].

As serotyping (H and K antigens) are not routinely performed after serogrouping (O antigen), it is possible that the presence or absence of toxin production of a serotype strain may not be representative of the whole serogroup. With the use of other diagnostic methods, like detection of toxin production and gene probing along with the conventional serogrouping method for the identification of EPEC resolves the confusion of toxin production by a serogroup. Further study is necessary to establish the relationship between different serotypes and toxin production by the EPEC strains.

2.5. EPIDEMIOLOGY OF EPEC

There is no doubt that the epidemiology and importance of *Escherichia coli*, as a cause of gastroenteritis, has increased greatly in recent years [Hart *et al.* 1993].

In the USA and Europe until about 1960, enteric *E. coli* was a common cause of epidemic diarrhoea within nurseries and children's institutions. In USA, from 1934 to 1987, there were over 50 outbreaks of infantile diarrhoea, 56% of which were due to EPEC serotypes [Moyenuddin *et al.* 1989]. Since 1960, the incidence and prevalence of diarrhoeagenic *E. coli* infections have declined dramatically in developed countries and at present, diarrhoea caused by EPEC is mainly sporadic in developed countries. By contrast, the incidence of enteropathogenic *E. coli* induced diarrhoea in children from developing nations (Africa, Far East, and South America) remains high. Isolation rates of enteropathogenic *E. coli* from these continents range from 4 to 37 percent (of all cases of enteritis), with most common serogroups being O26, O55, O86, O111, and O127. EPEC

is still often epidemic in the developing nations and it has been found to be responsible for many serious outbreaks of infantile diarrhoea throughout the world [Donnenberg and Kaper, 1992].

EPEC causes acute diarrhoea in the very young, rarely affecting children over 1 year of age and most closely associated with diarrhoea in those under 6 months [Gomes *et al.* 1991; Levine and Edelman; 1984, Regua *et al.* 1990].

Epidemiological studies of EPEC include the following studies of considerable importance, which are divided into two broad groups, one contains the case-control studies (Table-2.1), while the other one includes those studies performed without any control subjects (Table-2.2). In both the groups reports are listed in a chronological order mentioning the latest studies earlier to highlight the recent data.

Table-2.1 Case-control studies implicating EPEC as a cause of diarrhoea

Place	Author	Duration	% of cases	% of controls	P-value
Estail, Nicaragua	Mayapetek *et al*, 1993	1993	16	4	<0.05
Netherland	Rademaker *et al*, 1993	Oct'90-Apr'91	6.5	2.0	
New Caledonia	Begaud *et al*, 1993	1990	7.3	1.1	<0.05
Racife, Brazil	Magalhaes *et al*, 1992	Feb- June'89	37.8	13.5	
Sao Paulo, Brazil	Gomes *et al*, 1991	May'85-Jun'86	26	10	
Bangkok, Thailand	Echeverria *et al*, 1991	1988	7	3	=0.009
Sao Paulo, Brazil	Gomes *et al*, 1989	Feb'84-Mar'85	23	2	<0.0001
Seoul, Korea	Kim *et al*, 1989	Aug-Nov'86	6.5	4.8	NS
South Yugoslavia	Cobeljic *et al*, 1989	----------	7.9	4.2	<0.05
North India	Bhan *et al*, 1989	Nov'85-Jun'86	8.4	3.5	
Ghana	Agbodaze *et al*, 1988	----------	6.5	4	
West Germany	Karch *et al*, 1987	Oct'82- Sep'83	7.2	0.3	
Bangladesh	Moyenuddin *et al*, 1987		23.1	8.1	<0.01

*NS= not significant

Table-2.2 Studies without control subjects implicating EPEC as a cause of diarrhoea

Place	Author	Duration	Cases (%)
Israel	Lerman *et al*, 1994	Aug'88-July'92	6
Rio de Janerio, Brazil	Regua-Mangia *et al*, 1993	Jan'87-Feb'88	16.5
Singapore	Lim *et al*, 1992	Jan'90-June'91	2.7
Mosul, Iraq	Abbar *et al*, 1991	July'88-Feb'89	13.8
Tehran, Iran	Katouli *et al*, 1990	Feb'86-Mar'87	26.7
Bandar-Abbas, Iran	Katouli *et al*, 1988	March'84	31
Senegal	Darfeuille-Michaud *et al*, 1987	June'82-Dec'83	20.3

2.6. EPIDEMIOLOGY OF EPEC IN CHINA AND HONG KONG

The role of EPEC in diarrhoeal illness in China has also been reported. A case-control study conducted during April and May, 1989 has shown that EAF^+ EPEC was found in 5% of diarrhoea affected children and none of the matched controls [Kain *et al.* 1991]. An outbreak of EPEC O127:H6 diarrhoea was more recently reported from two nurseries for the new-born in Chong Qing, China in May, 1987 [Wu *et al.* 1992].

Although there is no case-control study in Hong Kong to demonstrate in the prevalence of EPEC in childhood diarrhoea, there have been studies indicating that EPEC is also responsible for diarrhoea in Hong Kong children. EPEC was shown to cause an outbreak of neonatal diarrhoea at a maternity hospital in 1982 [Yam *et al.* 1994].

A study performed between 1982 and 1985 in Queen Mary Hospital (QMH), of the University of Hong Kong. To identify EPEC this study tested 442 of a total 2,228 samples for EPEC by serogrouping only and 57 (12.9%) samples were positive [Lam *et al.* 1989].

Yam *et al* (1987) studied childhood diarrhoea in Hong Kong during 1982 to 1985 in Queen Marry Hospital and revealed that *E. coli* was associated with 23% of diarrhoeal episodes, of which 2.9% were confirmed to be of EPEC serogroups. Enterotoxigenic *E. coli* (ETEC) was diagnosed by laboratory methods in 0.9% cases. In the other 19% cases *E. coli* was isolated as a predominant or pure growth on repeated stool culture and no other conventional pathogens were isolated by the available methods.

2.7. CLINICAL INFECTION BY EPEC AND MANAGEMENT

2.7.1. Epidemiological syndromes

EPEC is a major causative agent of acute infantile diarrhoea and responsible for chronic or persistent diarrhoea as well [Clausen and Christe, 1982; Hill *et al.* 1991].

It also causes outbreaks of diarrhoea in infant nurseries including both sporadic and epidemic infantile diarrhoea in the communities. Furthermore, it has been implicated in endemic childhood diarrhoea in some countries, but rarely affects the adults.

2.7.2. Infective dose

From feeding experiments, it is known that infection can be initiated in adults with as few as 10^6 bacteria. Doses of 10^{10} bacteria have been successfully employed to infect all volunteers [Levine *et al.* 1978].

2.7.3. Incubation period

It has been observed that incubation period of EPEC varies widely from 3 to 60 hours [Donnenberg *et al.* 1993a; Hart *et al.* 1993].

2.7.4. Host factors

Outbreaks of EPEC diarrhoea in the community mainly occurred in the lower socio-economic groups. Perhaps the most notable feature of the epidemiology of EPEC diarrhoea is its predilection in nature for very young bottle-fed infants usually of less than six months [Regua *et al.* 1990; Abbar *et al.* 1991]. Infants of the second 6 months of life in developing countries are still commonly affected when they are being weaned from breast feeding. The association of the disease with bottle-fed infants and the sparing of breast-fed infants is an important factor to be considered. It has been shown in different studies that a decrease in relative frequency in isolation of EPEC with increasing age [Gomes *et al.* 1991; Levine and Edelman, 1984; Gurwith, MJ *et al.* 1977b].

2.7.5. Reservoirs of infection

Possible reservoirs of EPEC infection include:

1) Infants and young children with either clinical or asymptomatic infection;

2) asymptomatic adult carriers including mothers and persons who handle infants (older siblings, hospital personnel).

2.7.6. Routes of transmission

The precise mode of transmission of EPEC to young infants is not known. But it is thought to be primarily by faeco-oral route through contaminated food or faeces [Abbar *et al.* 1991]. Likely modes of transmission include direct contacts from contaminated hands, feeding bottles, weaning foods or formula, bathing equipment, even water and fomites. It may be also transmitted by airborne respiratory transmission and to the new-born during delivery as described by Bettelheim *et al* in 1974 [Wilkins *et al.* 1988].

2.7.7. Seasonal variation

In the earliest reports from Europe and the United States, EPEC were associated with infantile summer diarrhoea. Seasonality in the industrialised countries changed such that by the 1960s it was more often an infection associated with the cool season. In developing countries, EPEC infection still occurs more frequently in the warm season. In Brazil, EAF+ EPEC were detected frequently throughout the year, reaching the highest proportion in spring, when the weather was relatively dry and warming [Gomes *et al.* 1991].

2.7.8. Mechanism of diarrhoea

The manner by which EPEC strains cause diarrhoea is not fully elucidated, but the following factors, either alone or in combination, may contribute to produce diarrhoea:

(1) Extensive bacterial adhesion to the intestinal mucosa reduces the total absorptive surface area with diminished brush border enzymes causing malabsorption and osmotic diarrhoea.

(2) There may be an effect of as yet unidentified toxins, liberated by EPEC.

(3) The influence of inflammatory cells (in particular polymorphonuclear leukocytes) on intestinal function [Robins-Browne, 1987].

(4) It has been suggested that EPEC alters transcellular permeability, perhaps there by mediating secretion [Canil *et al.* 1993].

(5) Additionally, the increase in intracellular calcium concentration which accompany attaching and effacement of EPEC, could lead to chloride secretion and secretory diarrhoea [Bolton *et al.* 1977].

2.7.9. Histology

Jejunal biopsy shows moderate to severe villous atrophy with derangement of enterocytes. Electron microscopy further reveals dissolution of the glycocalyx and flattening of microvilli where bacteria adhere to enterocytes [Edelman and Levine, 1983].

2.7.10. Clinical features

Clinically, EPEC illness is characterised by low-grade fever, malaise, nausea, vomiting, abdominal cramps, and diarrhoea with prominent amounts of mucus, but without gross blood [Levine,1987]. Stool also may be watery and green coloured [Edelman and Levine, 1983]. Infants usually present with protracted diarrhoea and dehydration can be common feature as suggestive in a study, which demonstrated that as many as 71.6% cases were dehydrated [Regua Mangia *et al.* 1993]. Weight-loss and metabolic acidosis may also occur. Relapses and subsequent growth retardation are frequent sequlae [Hart *et al.* 1993; Edelman and Levine, 1983].

2.7.11. Treatment

As with all diarrhoeal diseases, correction with fluid and electrolytes balance is of prime importance. Many infants require intravenous fluid [Hart *et al.* 1993].

Very few controlled clinical trials have been performed to assess the role of antibiotics for the treatment of endemic EPEC gastroenteritis [de Olarte *et al.* 1974]. The role of antibiotics in treating endemic infantile diarrhoea caused by EPEC has not been determined. Thoren *et al* (1980) demonstrated in their study that in hospitalised children younger than two years with proven EPEC diarrhoea, general supportive therapy with fluid and electrolytes, along with antibiotics was superior to a regimen that did not include antibiotics. Both clinical and bacteriological cure on the 3rd day were found to be significantly higher in the antibiotic treated patients.

Aminoglycosides or polymixin B have been proven to be effective [Hart *et al.* 1993], but mecillinam or trimethoprim-sulfamethoxazole can also be used if the particular

EPEC strains are sensitive [de Olarte *et al.* 1974]. However, multi-resistant EPEC strains have already been reported by Gomes *et al* in Sau Paulo, Brazil [Gomes *et al.* 1991].

2.7.12. Prevention

There is no vaccine available for prevention [Hart *et al.* 1993]. However, Agbodaze *et al* (1988) demonstrated that improvement of sanitation lowers the incidence of EPEC diarrhoea and probably the nutritional status of both mother and baby has a role in determining the immunity against diarrhoeal infection [Abbar *et al.* 1991]. Breast-feeding can play a role in prevention of EPEC diarrhoea in young children [Fang, 1993]. Breast milk is thought to act by inhibiting the adhesion of EPEC to enterocytes by providing secretory IgA or an oligosaccharide-enriched fraction purified from colostrum and breast-milk [Cravioto *et al.* 1991]. Furthermore, other antibacterial factors in breast milk, such as cellular components, lactoferrin, etc. cannot be excluded as contributors to protection. Another mechanism for the protective effects of breast milk is its reduced environmental contamination. Formula and other foods have more possibilities to be contaminated during the preparation of milk or food.

2.8. DETECTION OF EPEC: LABORATORY METHODS

Since it is a normal intestinal flora, the definitive diagnosis of enteric *E. coli* infection is more difficult to establish than that of other enterobacterioceae, such as *Salmonella* or *Shigella*. In addition, serogrouping and virulence factors assays are usually not done routinely in the laboratories. The known laboratory methods for identification of EPEC infection are described briefly in the following.

2.8.1. O/H Serotyping

It is the oldest and most commonly used method in the laboratory for the detection of EPEC strains. In the 1940s, Kauffmann perfected a serotyping scheme for *E. coli* that was based on bacterial somatic (O), flagellar (H), and capsular (K) antigen. O antigens form the basis for the division of *E. coli* into serogroups. Within each serogroup, there are one or more serotypes that are based on the H antigen. It is done by agglutination with specific antisera.

2.8.2. Adhesion assay

EPEC strains attach to HEp-2 cultured cells in a distinctive manner producing localised adherence in tight clusters or microcolonies formation and it is commonly used for clinical diagnosis. Cravioto *et al* (1979) observed that unlike other *E. coli* categories, 80% EPEC strains adhered to HEp-2 cells in tissue culture and this can be seen by simple light microscopy.

Although detection of the LA pattern in the HEp-2 cell assay is a more practical method of identifying EPEC isolates, there is some limitations of the HEp-2 cell assay , because in a study the *E. coli* isolates in 3 of 14 patients classified as LA were enteroaggregative *E. coli*. This type of phenotypic misclassification in the HEp-2 cell assay results when very few cells show microcolonies or when two different phenotypes are seen in different fields of the same slide [Sharif *et al.* 1993].

2.8.3. EAF probe

Most of the EPEC strains show a characteristic localised adherence (LA) to HeLa cell and HEp-2 cell in culture. Baldini *et al* (1983) showed that LA is mediated by a ~60 MD-a plasmid. The plasmid was designated pMAR2 and the term EPEC adherence factor (EAF) was given to the gene product. Nataro *et al* (1985) first constructed a DNA probe from consisting of a 1-kb *BamHI-SalI* fragment of pMAR2, termed EAF probe, to detect EPEC strains with LA.

The probe has shown its usefulness in several studies world-wide, demonstrating 96-100% correlation between reactivity and the capability of a strain to cause LA [Echeverria *et al.* 1987; Levine *et al.* 1985]. A few strains which cause the attaching and effacing lesion are known to be negative by the EAF probe. The EAF probe is not likely to be available or feasible for use in most developing countries [Sharif *et al.* 1993], probably due to expenses and lack of necessary technical support.

2.8.4. FAS (Fluorescein Actin Staining) test

Knutton *et al* (1989a) have developed a highly specific method for identifying *E. coli* organisms that demonstrate attaching and effacing activity. This test uses fluorescein isothiocyanate (FITC) conjugated phalloidin, which specifically binds to the

filamentous actin, to identify the high concentration of actin in epithelial cells directly beneath areas of bacterial attachment.

The FAS test showed a high degree of sensitivity and specificity in detecting *E. coli* manifesting LA in the HEp-2 cell assay and is an improvement on the HEp-2 assay. FAS test avoids infrequent but nevertheless important phenotypic misclassifications in the HEp-2 cell assay and unlike in the HEp-2 cell assay even a few microcolonies can be easily detected in the FAS test because of the intense fluorescence in the apical surface at the site of concentration of actin. Furthermore, the FAS test always yielded clearly positive or clearly negative results. In laboratories where the *eaeA* (*E. coli* attaching and effacing) probe is not available, the FAS test may be useful as a confirmatory test for the identification of EPEC strains [Sharif *et al.* 1993].

The FAS test does not differentiate between AE (attaching and effacement) positive EPEC strains and AE positive EHEC verotoxigenic strains. Thus FAS testing in combination with verotoxin testing has been proposed as a more accurate method for detecting EPEC than O serogrouping [Smith *et al.* 1990].

2.8.5. ELISA (Enzyme Linked Immunosorbent Assay)

This was developed for detection of classical enteropathogenic *E. coli* serogroups. It detected EPEC positive for LA by HeLa cell assay and EPEC positive by EAF probe. A specific antiserum was raised with LA positive EPEC strain E2348/69 (serotype O127:H6) by immunising rabbits and then absorbing the antiserum with its LA negative derivative, MAR20. The absorbed antiserum reacted specifically with all 90 strains of *E. coli* belonging to eight different EPEC serogroups that were LA positive by HeLa cell assay. All EAF negative EPEC strains by DNA probe assay were also negative by ELISA. Thus the ELISA is 100% sensitive and specific in detecting LA^+ classic EPEC serogroups [Albert *et al.* 1991].

2.8.6. *eaeA* gene probe

EPEC is capable of attaching intimately to epithelial cells and effacing their microvilli. A chromosomal locus, *eaeA* (originally *eae*) is required for the intimate attachment to the intestinal cell wall.

A randomised double blind study performed by Donnenberg *et al.* (1993a) to determine the role of *eaeA* gene in human EPEC infection, which unambiguously assigns a role for *eaeA* as an EPEC virulence gene. In this study diarrhoea was developed in all 11 volunteers who received E2348/69 and in 4 of 11 who received the mutant (p=0.002). In another study this gene probe is found to be 100% sensitive and 98% specific in detecting *E. coli* of EPEC serogroups that demonstrate AE activity [Jerse *et al.* 1991b].

A second EPEC gene required for intimate attachment to epithelial cells is described very recently by some investigators. It is apparent from certain studies that the ability of EPEC to attach intimately to epithelial cells may be mediated by a gene cluster. The second gene implicated in the attaching and effacement is located immediately downstream of the *eaeA* gene locus, is defined as *eaeB* [Donnenberg *et al.* 1993b].

2.8.7. *bfpA* probe

EPEC express an inducible bundle forming pilus (BFP) associated with the presence of the EPEC adherence factor (EAF) plasmid and the ability for localised adherence (LA) on HEp-2 cells. The cloned structural gene (*bfpA*) encoding BFP was found only in EPEC and not in other enteropathogens. The *bfpA* probe was slightly more sensitive than the EAF probe. It may be a distinct advantage due to the fact that the *bfpA* probe contains sequences with a defined function, in contrast to the undefined function of the EAF probe sequences. Among EPEC strains with LA, the *bfpA* and EAF probes hybridised with 99% and 96% of the strains respectively [Giron *et al.* 1993a].

2.8.8. PCR (Polymerase Chain Reaction)

Recently PCR methods developed for the detection of EPEC harbouring a virulence plasmid like EAF, is named EAF-PCR. It appears to be a specific and efficient method for rapid detection of EPEC carrying EAF plasmids. From the EAF sequence, various oligonucleotides were constructed. The most specific primer pair used to detect EAF plasmid harbouring *E. coli* consisted of EAF21 and EAF25 [Jerse *et al.* 1990].

It has been observed that all EAF probe positive EPEC strains demonstrated positive PCR results. However, the results from 'Adherence assays' correlate less well with the PCR methods. Two strains which were EAF-PCR positive were found to be non-

adherent. If samples are screened by PCR, only positive, e.g. infectious strains have to be analysed by colony hybridisation for further to identification. PCR screening may be performed either directly on stool samples or with colonies grown on MacConkey agar plates [Franke *et al.* 1994].

A number of new techniques have been added to serology, the older method of *E. coli* classification. No doubt, our expanded understanding of the pathogenesis of *E. coli* diarrhoea has been assisted by recent advances in molecular biology.

Chapter-3

MATERIALS AND METHODS

3.1. PATIENT RECRUITMENT AND DATA COLLECTION

3.1.1. Study site

The study was conducted in the Prince of Wales Hospital (PWH) of Sha Tin in the New Territories of Hong Kong, one of the most rapidly developing industrialised countries of Asia. Prince of Wales Hospital is a large acute referral hospital containing 1,342 in-patient beds including 194 for paediatric admissions with specialist out-patient clinic and emergency service facilities. This hospital mainly serves the people of New Territories. It is the main teaching hospital of the Faculty of Medicine of the Chinese University of Hong Kong. In the year 1993, total 88,504 patients were admitted into the hospital (source: Hospital Medical Record Section) and 5,549 patients in the Paediatric wards. The paediatric unit has a separate ward for the patients with diarrhoea.

3.1.2. Study design

We set out to use a hospital-based prospective case-control study design to assess the prevalence of EPEC and other enteric pathogens in childhood diarrhoea in Hong Kong. An analysis of the clinical features of different pathogens was also undertaken. Patients admitted in the paediatric gastroenteritis ward in Prince of Wales Hospital with a diagnosis of acute diarrhoea were eligible for selection as cases. Controls were selected from paediatric patients admitted to PWH without diarrhoea. Stool specimens were collected from both the groups and initially examined with the existing laboratory facilities in the Department of Microbiology in Prince of Wales Hospital. Colonies from MacConkey agar morphologically resembling to *E. coli* were saved for further analysis.

We had a plan to screen out all the presumptive *E. coli* colonies by a Rosco Double-Test Tablet [Dominguez *et al.* 1992] before further analysis by serogrouping, other specific tests related to EPEC (e.g. FAS test, HEp-2 cell adherence test) as well as by gene probing. Rosco Double-Test along with serotyping and some other important tests for the identification of EPEC were not performed finally due to lack of financial support. The saved *E. coli* colonies were further analysed into different categories by gene probing in the Department of Bacteriology, Immunology and Molecular Genetics of the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok.

3.1.3. Study period

Before starting the patient recruitment, the nurses working in paediatric wards and the technicians working in the Bacteriology and Virology laboratories were informed regarding the methods and objectives of the study. Recruitment of patients for the study was carried out over a period of one year, from August, 1994 to July, 1995. A further 8 weeks was spent for preliminary data analysis and gene probing of the saved *E. coli* colonies. Final analysis was then performed with the results of genetic analysis.

3.1.4. Study population

Prince of Wales Hospital is a large government hospital which provides heath care to New Territories, with a current estimated population of about 1,085,000. Our study population includes all patients of paediatric age group (0 to 15 years) admitted to PWH. Most of the patients were Chinese in origin and a few were Vietnamese, who were residing in the detention centres of that area during the study period.

3.1.5. Selection of patients

In this study, two groups of patients were selected, maintaining the criteria of patient selection, separately for the case and the control group. The patients were selected mainly in working days. But the patients admitted on a holiday were also recruited on the next working day if the patient fulfilled all the criteria for the study as described below.

3.1.6. Inclusion criteria for cases

All children admitted to the gastroenteritis ward at PWH with a clinical diagnosis of acute diarrhoea were eligible for admission to the study. Acute diarrhoea was defined as 'an increase in stool frequency by 3 or more unformed stools in the previous 24 hours for a period of less than 14 days' [Echeverria *et al.* 1991].

3.1.7. Exclusion criteria

a) Children with a history of previous episodes of diarrhoea or antibiotic therapy within last 2 weeks period before admission to hospital.

b) Children with a history of recent, significant other gastrointestinal diseases, e.g. cow's milk protein intolerance, inflammatory bowel diseases, abdominal irradiation for treatment of malignancy.

c) Children with known immunodeficiency states, and

d) Failure to pass a loose stool within 48 hours of admission or discharged before any bowel evacuation.

3.1.8. Selection of control group

Control subjects were selected from the in-patients admitted to the paediatric wards without diarrhoea. It was initially planned to select a control matched with each case within two weeks of admission of the case. The criteria for matching were sex and age. According to this plan, the matched control should be of the same sex of a particular case. To match with age we categorised the whole age range (from birth to 15 years) of our cases into different categories, as follows: up to the age of one month, 2-4 months, 5-12 months, 13-24 months, 25-60 months and 61 months or above. The following reasons were considered for selecting these age groups: neonates, infants up to 1 month of age, are susceptible to certain specific pathogens. We made 2-4 months a separate category, as after four months mothers are often advised to start weaning their children. So it may be possible to identify the pathogens which are particularly transmitted through weaning food. Most commonly the children suffer from diarrhoeal diseases due to EPEC infection below two years of age [Edelman and Levine, 1983]. We categorised the children up to two years into another two groups to identify the age importance in diarrhoea in relation to different causative pathogens. After two years the children begin to come out of the

house in contact of other children in play ground or in the Kindergarten. We limited this age group up to 5 years to cover the pre-school period. Diarrhoea is less frequent in the older children above five. So we did not further categorise this age group (6-15 years).

But over a month of pilot study we faced a lot of problems in implementation of the initial design of selecting matched controls. The main difficulties related to collection of stool samples from the selected controls were-

1. Over three quarters of our initially selected controls and a significant number of cases failed to pass stool within 48 hours of admission or over the period of their hospital stay (when stay <48 hours).

2. Furthermore, there was not available man power to collect stool samples from their houses once the control subjects were discharged from the hospital.

So, we had to change our matching criteria and finally we decided to recruit the unmatched controls. During the selection of unmatched control, we stratified as far as possible within the various age categories as defined for the diarrhoea patients, as diarrhoea was relatively more commonly seen in the younger age group. The exclusion criteria were same for the cases and controls.

3.1.9. Collection of stool specimens

The cases, after recruitment into the study, were immediately designated with a study number. The identification of the patient was entered in the record book. Then the on-duty nurse was advised to collect stool samples from every selected patient. Stool specimens were sent for bacterial culture and sensitivity, microscopic examination of ova and cysts of the parasites, and ELISA (Enzyme linked immunosorbent assay) for rotavirus.

Each of the control subjects was also designated with a survey number and identification was entered in the record book as for the study patients. However, only one stool specimen was collected from each patient and this was sent for bacterial culture and sensitivity, microscopic examination of ova and cysts of parasites.

We tried to collect the first stool specimen after admission and all stools were collected from both the groups within 48 hours of hospitalisation to avoid nosocomial infection. After collection of stool specimen in a sterile container, it was sent to the laboratory. When transport was delayed overnight, the specimens were kept at 4^0 C in the refrigerator. Once a stool specimen was sent, the on-duty nurse was asked to put a tick ($\surd$) mark in a specific

area of the record book, so that we could monitor the collection of specimens. The ordinary requisition forms for the investigations were clearly identified by using a rubber stamp for our study to distinguish the specimens from the others [See Appendix-7.3].

A significant number of patients (112) admitted with a diagnosis of gastroenteritis were enrolled into the study but subsequently failed to pass any stool. These patients were excluded from the study and new patients were recruited against the study numbers. The control subjects who failed to give one stool sample within first 48 hours of admissions, were also removed from the study and new controls were recruited against those particular numbers. This failure in passing stool might have been due to a) a short duration of hospital stay, b) infrequent bowel evacuation was their normal habit in fact, or c) as a result of inactivity during the illness.

3.1.10. Treatment of the study-patients

The patients were treated as usual and our study at no time interfered with the scheduled treatment proposed by the respective responsible physician. If any subject was prescribed antibiotic therapy, no sample was collected after commencement of antibiotics.

3.1.11. Collection of data

Data were collected from every patient on a specific data-form prepared by and piloted before study commencement [see Appendix-7.1]. The data-form contained particulars of the patient identifies, medical history, social history and family history. Physical and laboratory investigation data were also recorded. Data on the children were collected from two sources:

1) Clinical information and other history were abstracted mostly from the child's case note and physical examination on admission performed by the attending Medical Officer (MO). Vital signs (pulse-rate, temperature and rate of respiration) data were collected from the nurses' records.

2) Medical history and social data were also collected directly from the child's mother or primary care giver (e.g. grandmother) by interview. This was possible as a result of the help of a cantonese speaking research nurse who was able to spend an estimated 5 hours a week in the initial period of the study (August, 1994 to January, 1995). However, it was difficult for her to meet with all the parents or other primary care-givers, as most of

the visitors attended their children after office-hours. After her departture in February,95 data were collected with the help of cantonese speaking medical students. Unfortunately in 10% of patients data collection was incomplete resulted in some missing data. We tried, where possible, to abstract these data from case notes prepared by the Medical Officer.

3.1.12. Ethical approval

The study was ethically approved by Clinical Research Ethics Committee of the Chinese University of Hong Kong.

3.2. LABORATORY METHODS

This study was assisted by the Department of Microbiology in Prince of Wales Hospital, and also by the Department of Bacteriology, Immunology and Molecular Genetics of Armed Forces Research Institute of Medical Sciences in Bangkok.

The laboratory methods followed in the Microbiology laboratory in PWH were according to guide for the laboratory staff prepared by the Department of Microbiology. The Methods followed in AFRIMS laboratory was according to the methods for detection of diarrhoeagenic *E. coli* by using Nucleotide Probes, prepared by Echeverria *et al.* (1990).

3.2.1. IN PWH LABORATORY

Like all other specimens, stool samples from our cases and controls were received in the laboratory reception section of the Department of Microbiology. Each of the samples was then marked with specific laboratory identification number in the reception as a routine procedure. Specimens for rotavirus assay were distributed to the virology section and the others were sent to the stool bench for processing. Among the specimens of the stool bench, those were specially indicated for our (EPEC study) study, were analysed as follows:

1. Routine culture and sensitivity tests were performed for commonly isolated bacterial enteropathogens (*Salmonella, Campylobacter, Shigella and Aeromonas*). These samples were also examined under microscope for ova and cysts. This part of work was carried out by the laboratory staff. Laboratory procedures are described in the Appendix-7.2.
2. When the laboratory staff finished their work, the samples were kept on the table allocated for me in the laboratory. Further culture for *E. coli* with these specimens were performed by me.

a) Isolation of E. coli

Initially for the isolation of *E. coli,* every specimen was inoculated directly onto MacConkey agar plates and incubated overnight at a temperature of 37^0C for a period of 18-24 hours. Those plates demonstrated either pure or predominant growth of lactose-fermenting and also non-lactose fermenting colonies morphologically resembling *E. coli,*

were selected for further testing. The colonies were identified solely by the morphological appearance. They are of 2-4 mm in diameter, smooth, glossy, convex with an entire edge and are usually rose-pink but may be pale in colour in the case of non-lactose fermenting *E. coli*. Ten single selected colonies from each plate were picked and individual colony was preserved into separate nutrient agar slopes. Nutrient agar slopes were incubated overnight at 37^0C and then stored in room temperature for further analyses.

b) Materials used

1. MacConkey agar: It was used as the selective medium for the growth of *E. coli*. The method of preparation of this medium was as follows:

50 grams of MacConkey agar was diluted in one litre of distilled water, then it was mixed, boiled, and autoclaved at 125^0C for 15 minutes. After allowing to cool (about 50^0C) the media was distributed into plates, using about 20 ml per plate.

2. Nutrient agar: This medium was used for the preservation of the *E. coli* colonies. In 3 litres of Nutrient Agar (NA) base, there were Lab-lemco, Peptone and Agar (30 gm of each). For use, 3 litre nutrient agar base was mixed with 3 litres of water at 121^0C for 15 minutes and the final pH was 7.4.

3.2.2. IN AFRIMS LABORATORY

Dr. Peter Echeverria, Chief of the Department, kindly agreed to teach me the techniques of gene probing for the isolation of different categories of *E. coli*, particularly EPEC. Saved colonies of *E. coli* were carried for gene probing to the Department of Bacteriology, Immunology and Molecular Genetics at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok in Thailand. AFRIMS is a research institute of the US army, and has the available latest technology for research into enteric pathogens. In the AFRIMS laboratory, a total of eight polynucleotide gene probes (LT, STIa, STIb, EAF, *eae*A, *bfp*A, DA and EAgg) were used for identification of EPEC, ETEC, EAggEC and DAEC (Diffusely adherent *E. coli*). Investigations for EIEC and EHEC were not performed.

Polynucleotide probes are endonuclease digestion fragments of plasmids containing cloned enterovirulent determinants. The probes include several steps as outlined below:

I. Plasmid purification of cloned DNA that contain a specific virulent determinant.

II. Endonuclease digestion of plasmid DNA (Elution and concentration steps).

III. Spotting and processing of bacterial colonies on filter membranes.

IV. Preparation of ^{32}P labelled probe by either Nick Translation or Random Priming.

V. Hybridization assay.

VI. Autoradiography.

Out of the above mentioned 6 steps, the initial 2 steps were performed by the laboratory staff. I started my work from step-III (spotting of the colonies) up to the end of the process. Each step will be now discussed in detail.

I. Plasmid purification of cloned DNA that contain a specific virulent determinant

a) Storage and selection of clones

Escherichia coli K-12 containing the cloned enterovirulent determinants were kept at -70^{0}C in skim milk media. They were inoculated on to Mueller-Hinton agar. An antibiotic disc (Ap = Ampicillin) was placed on the agars. The agar and disc were incubated at 37^{0}C overnight. This was to select for isolates that contain the plasmid with the cloned determinants.

Table- 3.1 Plasmids and references of each probe

Determinant	Plasmid	Disc	References	broth (μg/ml)
LT	pEWD 299	Ap	Dallas & Falkow, 1979	50
STIa	pDAS 101	Ap	Sommerfelt et al. 1988	50
STIb	pDAS 100	Ap	Sommerfelt et al. 1988	50
DA	pSLM 852	Ap	Bilge, S.S. et al.1989	50
EAF	pMAR 22	Ap	Nataro et al.1985b	50
eaeA	pCVD 434	Ap	Jerse, A.E. et al.1991b	50
EAgg	pCVD 432	Ap	Baudry, B. et al.1990	50
bfpA	pMSD 207	Ap	Giron et al. 1993a	50

b) Plasmid purification

Plasmid of cloned virulent determinant can be purified using large scale Birnboim method as follows:

1. A single colony selected from antibiotic plate was inoculated into 6 ml of L-broth (pH 7.5) with antibiotics. Tubes were shaken overnight at 37^0C.
2. 5 ml of the growing culture was then inoculated into 500 ml L-broth with antibiotics correspond to each cloned bacteria (see table-3.1) and was shaken at 37^0C until the density of cells was increased >5 X.
3. Chloramphenicol was added to give a concentration of 150 μg/ml and the flask was then shaken overnight for 16 hours.
4. This was then centrifuged at 8,000 g for 15 minutes at 4^0C (6,000 rpm in a GSA head of a Sorvall centrifuge).
5. Bacteria were collected from the tubes after centrifugation and were washed in TES (10 mM Tris.Cl, pH 8.0 + 1 mM EDTA, pH 8.0 + 0.1 m NaCl).
6. The bacteria were resuspended in 40 ml of Solution I, inverted gently, and incubated at 0^0C for 5 minutes.
7. 80 ml of Solution II was added, inverted gently, and incubated at 0^0C for a further 5 minutes.
8. 60 ml of Solution III was added, inverted gently, and incubated for >1 hour at 0^0C.
9. It was centrifuged at 140,000 g for 15 minutes at 4^0C and supernatant was retained.
10. Two volumes of 95% ethanol were added and incubated for 1 hour at -70^0C to precipitate the plasmid DNA.
11. It was centrifuged at 140,000 g for 20 minutes at 4^0C and dissolved the pellet in 27 ml of TES (Tris, EDTA, Sodium-Chloride).
12. 25 g CsCl (Cesium chloride) was added and mixed gently, until it was dissolved in solution.
13. Density was adjusted to 1.58-1.60 with additional CsCl in TE (Tris-EDTA). This was done by weighing 10 ml of the suspension.
14. It was transferred to VTi 50 or Ti 60 tubes (VTi and Ti, these are tubes of different sizes).
15. 1 ml of ethidium bromide was added and mixed by inversion.

16. It was centrifuged at 40 K for 40 hours (60 Ti) or 50 K for 12 hours (VTi 50).

17. The sealed tubes were vented with an 18-gauge needle and the lower band was then collected with a syringe.

18. Ethidium bromide was extracted with isopropsnol saturated with 5 M NaCl in TE four times (one time after the red colour disappears).

Details of the media and solutions used at different steps of plasmid purification of cloned DNA are described in the following section.

3.2.A. MEDIA

1. Skim milk media

This is a standardised product recommended for culture media. When prepared in 10% solution it is equivalent to fresh skim milk. To rehydrate the medium, 100 g of bacto-skim milk (commercial preparation) is dissolved in 1000 ml freshly distilled water and sterilised in the autoclave for 15 minutes under 15 lb pressure at 121^0C.

2. Mueller-Hinton agar

Per litre

Beef, infusion from------- 300 gm

Bacto Casamino acids---- 17.5 gm

Starch---------- 1.5 gm

Bacto-agar---------- 17 gm

38 gm was suspended in 1 litre distilled water and boiled to dissolve completely. It was sterilised at 121-124^0C for 15 minutes. Final pH was 7.3±0.1 at 25^0C.

3. L-Broth media (Luria-Bertani Medium)

Per litre

To 950 ml of deionised H_2O,

bacto-tryptone----------10 g

bacto-yeast extract-------- 5 g, and

NaCl----------10 g

were added and was shaken until the solutes were dissolved. pH was adjusted to 7.0 with 5N NaOH (~0.2 ml) and volume of the solution was made 1 litre with the addition of deionised H_2O. It was sterilised by autoclaving for 20 minutes under a pressure of 15 lb/sq.inch on liquid cycle.

3.2.B. SOLUTIONS

1. Solution I (hold at 0^0C)

50 mM glucose

10 mM EDTA

25 mM Tris HCl, pH 8.0

5 mg/ml lysozyme

For 5 ml of working solution :

250 µl of 1 M glucose (sterilised by 0.45 µmillipore membrane)

50 µl of 1 M EDTA (sterilised by autoclaving)

125 µl of 1 M Tris HCl (sterilised by autoclaving) and

0.025 g of lysozyme (kept at 4^0C), was made up to 5 ml by DDW.

2. Solution II

0.2N NaOH and 1% SDS

Working solution

2 ml of 0.4 N NaOH (sterilised by autoclaving)

2 ml of 2% SDS (sterilised by autoclaving)

Stored at room temperature (stable for approximately 1 week)

3. Solution III

3 M NaOAC (Sodium acetate), pH =4.8

Anhydrous NaOAC was dissolved in minimal volume of distilled water. It was titrated to pH 4.8 with glacial acetic acid and was brought to full volume and stored at 4^0C.

4. 1 M Tris (pH 8.0)

121.1 g Tris base was dissolved in 800 ml of distilled water. The pH was adjusted at 8.0 by adding approximately 42 ml of concentrated HCl and was sterilised by autoclaving.

5. TE buffer (pH 8.0)

10 mM Tris.HCl (pH 8.0)

1 mM EDTA (pH 8.0)

6. 0.01 M EDTA (pH 8.0)

3.722 g of EDTA.$2H_2O$ was added to 800 ml of distilled water and was stirred vigorously on a magnetic stirrer. pH was adjusted to 8.0 with NaOH (0.4 g NaOH pellets) and was sterilised by autoclaving.

7. 0.7% Agarose

1.4 g agarose was boiled in TE (Tris + EDTA) buffer with magnetic stirrer.

TE, pH 8.0 is a mixture of 10 mM Tris.Cl (pH 8.0) and 1 mM EDTA (pH 8.0).

8. 10 X TB (Tris-boric)

108 g of trisma base, 55 g of boric acid and 9.3 g of EDTA di-sodium salt was dissolved in DDW (Double distilled water). pH was adjusted to 8.3 and was made it up to 1 litre.

9. Ethium bromide (10 mg/ml)

1 g of ethium bromide was added to 100 ml of DDW and stirred on a magnetic stirrer for several hours to ensure that the dye was dissolved. The container was wrapped in aluminium foil and stored at 4^0C.

II. Endonuclease digestion of plasmid DNA

1. 1 μg of plasmid DNA was digested with 1 unit of the particular endonuclease in the appropriate buffer at the indicated temperature (usually 37^0C) for 1 hour, and then it was heated at 65^0C for 10 minutes.

2. The digested DNA was separated by electrophoresis on the agarose gel in Tris-borate buffer.

DNA fragments separated by electrophoresis were cut out of gel with a razor blade and electrolute using Isco-electrophoretic concentrator by the following procedure:

a. DNA band was cut into fine pieces.

b. The chamber of Isco-electrophoretic concentrator was filled in.

c. Buffer was added as the following:

- 75 ml of buffer III (3 M Sodium acetate is prepared in 1:2 dilution of buffer II) in compartment A at the same time of 90 ml of buffer III in compartment B.
- 90 ml of buffer II (100 mM Tris and 0.2 mM EDTA, adjusted to pH 7.8, and stock buffer was prepared 10X) in compartment C at the same time of 75 ml of buffer III in compartment D.

d. The electric current (1 watt) was applied for at least 2 hours, pipette buffer out of wells and concentrated DNA (approximately 200 ml) was collected into 15 ml microcentrifuge tubes.

e. Cold 3M NaOAC (408.1 g of sodium acetate.$3H_2O$ was dissolved in 800 ml of DDW and pH was adjusted to 5.2 with glacial acetic acid. The volume was made up to 1 litre and sterilised by autoclaving) pH 5.2 was added, approximately 20 µl (0.1 X DNA volume).

f. Cold ethanol 440 µl (2 X of total volume) was added and gently mixed.

g. It was kept at -20^{0}C overnight.

h. It was centrifuged at 10,000 rpm for 10 min.

i. Pellet in TE was resuspended to get a high concentration and to determine the concentration by running electrophoresis compared to marker size standards.

Table-3.2 Endonucleases and size of the fragments

Determinant	Plasmid	Endonucleases	Agarose	Fragment bp (base pair)
LT	pEWD299	Hinc II	0.8%	850
ST Ia	pDAS101	EcoRI/ BamHI	1.2%	157
ST Ib	pDAS100	BamHI/ Pst I	1.2%	215
DA	pSLM852	Pst I	1.2%	450
EAF	pMAR22	BamHI/ Sal I	0.8%	1000
eaeA	pCVD434	Kpn I/ Sal I	0.8%	1000
EAgg	pCVD432	EcoRI/ Pst I	0.8%	800
bfpA	pMSD207	EcoRI	0.8%	850

III. Spotting and processing of bacterial colonies on Whatman filter No. 541

The samples were numbered and 10 colonies in nutrient agar slopes for each patient were designated by numbers 1-10 for identification of the specific colony. The template was designed so that one MacConkey agar plate can contain 36 spots for subculture (10 picks from each of the 3 patients plus 5 positive controls for 5 probes and one negative control). The positive and negative controls were subcultured everyday. All the bacterial strains used as controls for gene probing were kindly provided by Dr. Peter Echeverria (AFRIMS, Bangkok).

The cloned bacteria used for different controls were, pCVD 432 for EAgg, pSLM 852 for DA (Diffusely Adherent), pMSD 207 for *bfpA*, pMAR 22 for EAF, AS-04-1 for wild type *eae* strain and Xac for *E.coli* K-12 (negative control) probes.

MacConkey agar plates were dried in the incubator for 15 minutes and then enumerated for identification. Isolates from the nutrient agar slopes and the controls were inoculated over the specific area on the MacConkey agar plate. The plate was incubated at 37^0C overnight. On the next day, the plates were examined for growth and findings were recorded in the record book (see figure- 3.1).

Then Whatman 541 filters (7 cm diameter) were enumerated as like as on the MacConkey plate and pressed evenly over the bacterial growth. 541 filter was lifted in a

glass petridish on Whatman 3 paper saturated with Solution-A (a mixture of 0.5 N NaOH and 1.5 M NaCl, 20 g of NaOH and 87.75 g of NaCl were dissolved in DDW). Then the glass-plates were covered and boiled in the microwave oven under high power for 2 minutes (as we used 20 petridishes, 1 minute for 10 petridishes). Whatman 541 filter was separated from the Whatman 3 papers and immersed in solution-B (a mixture of 1 M Tris and 2 M NaCl, 121.1 g of Tris and 117 g of NaCl were dissolved in DDW and pH was adjusted to 7) for 4 minutes and air-dried. Whatman 541 on paper towel and was kept at room temperature in a dry, cool place until further use.

Figure- 3.1 Findings of subculture done by spotting onto MacConkey agar medium

IV. Preparation of ^{32}P labelled probes

DNA fragments were labelled by Random Priming method to incorporated ^{32}P nucleotides.

Random Priming Method

1. 25 ng of denature DNA was dissolved in 5-20 µl of dilute buffer in a microcentrifuge tube by heating for 5 minutes in a boiling water bath, then immediately cooled on ice.
2. The following reagents were added on ice:

   1 µl d ATP (deoxy Adenosine tri-Phosphate) solution

   1 µl d GTP (deoxy Guanine tri-Phosphate) solution

   1 µl d TTP (deoxy Thiamine tri-Phosphate) solution

   20 µl Random primers buffer mixture

   5 µl ^{32}P d CTP (deoxy Cytosine tri-Phosphate), 3000 Ci/ mmole; 10 UCi/ ml.

Distilled water was added up to a total volume of 49 µl.

3. It was mixed briefly and 1 µl of klenow fragment was mixed gently but thoroughly and centrifuged briefly.
4. It was incubated at 25^0C for 1 hour.
5. 5 µl stop buffer was added.
6. Sephadex column (Select D G-50 5'-->3' company) was set at room temperature.
7. Caps were removed and centrifuged at 2,000 rpm for 3 minutes.
8. Labelled DNA was put on the surface of the column.
9. It was centrifuged at 2,000 rpm for 4 minutes and supernatant fraction was collected.
10. Radioactivity was counted of 1 µl fraction and kept at -20^0C until further use.

V. Hybridization Assay for DNA labelled probes

a. Prehybridization

The membrane filters were soaked in prehybridization buffer for 1-3 hours at 42^0C.

The prehybridization buffer contains 10 X Denhardt's solution, 4 X SET pH= 8.0.

0.5% SDS (Sodium dodecyl sulphate) and 10 µg/ml CTD (Calf thymus derivative).

50ml/ 50 filters

100 X Denhardt's solution (see below)	5.0 ml
20 X SET (NaCl, EDTA,Tris)	10.5 ml
20% SDS	1.25 ml
10 mg/ml CTD (denatured)	0.5 ml
H_2O	33.25 ml
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Total volume	50.0 ml

20% SDS was added after the addition of H_2O to prevent precipitation. CTD (Calf thymus DNA) was boiled in boiling water for 10 minutes and cooled on ice bath before use.

b. Hybridization

After prehybridization, the solution was poured off and all the filters were put in another bag. The hybridization buffer are the solution containing 50% Formamide, 2 X Denhardt's solution, 4 X SET, 0.4% SDS, 6% PEG (Poly ethylene glycol), 50 μg/ml Heparin and 200μg/ ml CTD.

100% Formamide	25.0 ml
100 X Denhardt's solution	10.0 ml
20 X SET	10.0 ml
20% SDS	1.0 ml
10 mg/ml CTD (denatured)	1.0 ml
40% PEG	7.5 ml
50 mg/ml Heparin	0.5 ml
---	---
Total	50.0 ml

The labelled DNA was added to the hybridization buffer. The amount of labelled DNA was calculated to about 2 X 10^6 cpm per 10 ml of buffer. In short, DNA fragment probe such as STIa and STIb probe, it was recommended that 4 X 10^6 cpm per 10 ml buffer should be added to the hybridization buffer.

c. Post-hybridization assay

1. Washing solution 2,000 ml/ 100 filters was prepared.
2. The filters were washed in 2 X SSC/ 0.2% SDS for 10 min at room temperature and once repeated.
3. The filters were washed in 2 X SSC/ 0.2% SDS for 10 minutes at 65^{0}C and twice repeated.
4. The filters were washed in 0.1 X SSC/ 0.1% SDS for 15 minutes at 65^{0}C and once repeated.
5. The filters were ringed in 2 X SSC at room temperature for 10 minutes.
6. The filters were then air-dried.

The following buffers were used in the preparation of ^{32}P labelled DNA (17kb) probe:

3.2.C. BUFFERS FOR PREPARATION OF ^{32}P LABELLED DNA (17kb) PROBE

1. 0.5 M EDTA (pH 8.0)

186.1 g of EDTA.2H_2O was added to 800 ml of DDW and was stirred vigorously on a magnetic stirrer. pH was adjusted to 8.0 with NaOH (20 g of NaOH pellets) and sterilised by autoclaving.

2. Denhardt's solution (100 X)

Ficoll	10 g
Polyvinylpyrolidone	10 g
BSA (Pentax Fraction V)	10 g
H_2O was added up to	500 ml.

It was filtered through a disposable Nalgene filter. It was dispensed into 25 ml aliquots and stored at -20^{0}C.

3. SET (Sodium Chloride, EDTA and Tris) [20 X]

30 mM Tris, pH 8.0

150 mM NaCl

1mM EDTA

4. 20% SDS (Sodium dodecyl sulfate)

200 g of electrophoresis grade SDS was dissolved in 900 ml DDW and heated to 68^0C to assist dissolution. pH was adjusted to 7.2 by adding a few drops of concentrated HCl and volume was made up to 1 litre.

5. 20X SSC (Sodium citrate, sodium chloride)

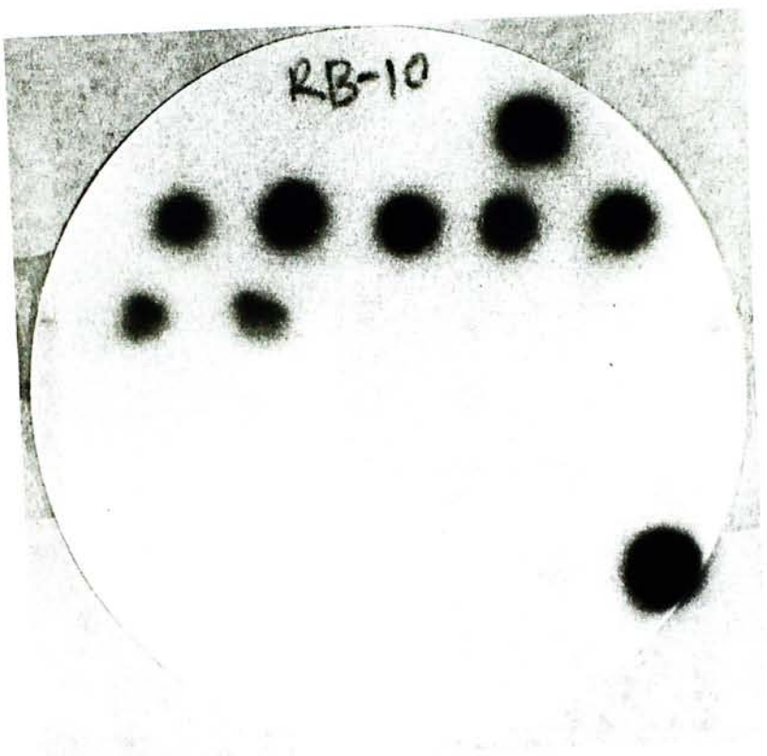
175.3 g of NaCl and 88.2 g of sodium citrate was dissolved in 800 ml of DDW. pH was adjusted to 7.0 with a few drops of 10N solution of NaOH and volume was made up to 1 litre and was sterilised by autoclaving.

VI. Autoradiography

1. The filters were attached on a sheet of paper with tape and was put in a film holder.
2. An X-ray film (X-Omat AR) was placed in between the paper sheet and intensifying screen.
3. It was incubated at -70^0C overnight.
4. The film was developed according to the manufacturer instruction.

Result was read from the X-ray film comparing the shadow of the specimen with that of the control. Positive reaction with one probe is shown in figure- 3.2

Figure-3.2 Positive reaction with EAF (EPEC Adherence Factor) probe



REUSE OF FILTERS

After using once we washed the filters with a mixed solution of 0.5 N NaOH and 2 X SSC, 0.1% SDS and used the filters again for some other probes (LT, STIa and STIb). The positive controls we used for LT, STIa and STIb were pEWD 299, pDAS 101 and pDAS 100 respectively.

3.3. DATA MANAGEMENT AND STATISTICAL METHODS

Data was entered into the computer through a data-file prepared by the Epi info-6 programme (CDC, Atlanta). Statistical analysis was done using Epi info package available through the Department of Paediatrics, CUHK.

Chapter-4

RESULT

A total of 388 cases and 306 controls were studied during a one year study period, from August, 1994 to July, 1995. These controls were unmatched but selected to be within of a similar age range of the cases. They were selected from children admitted to Prince of Wales Hospital without diarrhoea.

4.1. DEMOGRAPHY OF THE PATIENTS

4.1.1. Age distribution of the patients

The age distribution of cases and controls is shown in table- 4.1.

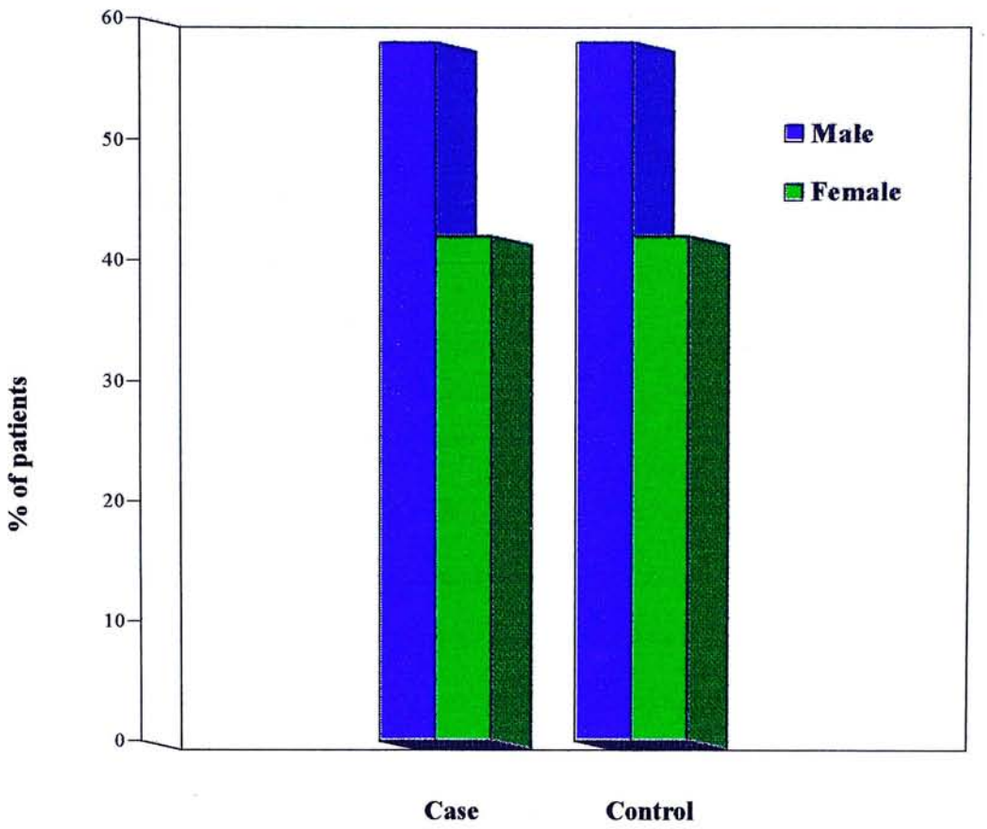
Table- 4.1 Age distribution of the patients

Age distribution	Cases (%) (n=388)	Controls (%) (n=306)
Birth - <3 months	69 (17.8)	36 (11.8)
3- <6 months	45 (11.6)	37 (12.1)
6- <12 months	97 (25)	64 (20.9)
12- <24 months	96 (24.7)	67 (21.9)
24- <60 months	62 (16)	79 (25.8)
60 months and above	19 (4.9)	23 (7.5)

4.1.2. Sex distribution of the patients

Sex distribution in the cases and controls were comparable. Among the cases, 58.2 % were males and male: female ratio was 1.39: 1. The male-female ratio in the control group were very similar to that of the cases (1.4:1). These results are presented in figure- 4.1.

Figure- 4.1 Sex distribution of the patients



4.1.3. Ethnic origin of the patients

In Hong Kong, over 99% of the population are ethnic Chinese (Department of Health, Hong Kong, 1993-94). In our study, over 92% of the cases were Chinese and 7% were Vietnamese who were living in the detention centres during the study period. A small number of patients were Caucasian and Filipino. The majority of the controls were Chinese with a smaller number of Vietnamese children. Ethnic origin of the patients are shown in the table- 4.2.

Table-4.2 Distribution of ethnic origin of the patients

Ethnic origin	Cases (%) (n=386)	Controls (%) (n=291)
Chinese	353 (91.5)	283 (97.3)
Vietnamese	26 (6.7)	8 (2.7)
Caucasian	6 (1.5)	0
Filipino	1 (0.3)	0
(Missing data	2	15)

4.1.4. Distribution of area of abode in Hong Kong

The study was conducted in Prince of Wales Hospital which mainly serves the people of the New Territories of Hong Kong. Well over 90% of the cases and controls were residing in the New Territories. About 2% of the cases were residents of mainland China and were admitted during a visit or were brought to Hong Kong because of the illness. This result is presented in the table- 4.3.

Table- 4.3 Distribution of area of abode

Area	Cases (%) (n=350)	Controls (%) (n=287)
New Territories	332 (94.8)	281(97.9)
Kowloon	8 (2.3)	4 (1.4)
China	7 (2.0)	0
Hong Kong Island	3 (0.9)	2 (0.7)
(Missing data	38	19)

4.1.5. School attendance of the patients

As most of our patients in both cases and controls were very young, 87.4% of cases and 80% control were too young to go to Kindergarten. Only 4.1% cases and 4.8% of controls were studying in schools. 8.5% of the cases and 15.2% of controls were students in Kindergarten. The result are shown in the table- 4.4.

Table-4.4 School attendance of the patients

Educational status	Cases (%) (n=388)	Controls (%) (n=290)
School	16 (4.1)	14 (4.8)
Kindergarten	33 (8.5)	44 (15.2)
Neither	339 (87.4)	232 (80.0)
(Missing data	0	16)

4.2. PREDISPOSING FACTORS FOR DIARRHOEA

4.2.1. History of breast feeding of the patients

Parents were asked if the child had 'ever been breast-fed'. Only 3% of children were still breast feeding during the study period. The ever breast feeding rate in the cases and controls were 12.6% and 6.7% respectively. The data for breast feeding are shown in the table- 4.6

Table-4.6 Rate of breast feeding among cases and controls

Group	Cases (%) (n=356)	Controls (%) (n=267)
Ever breast-fed	45 (12.6)	18 (6.7)
Currently breast-fed	10 (2.8)	4 (1.5)
Never breast-fed	311 (87.4)	249 (93.3)
(Missing data	32	39)

Relative incidence of the rate of ever breast feeding among the children of different ethnic origin has been shown in the following table- 4.7

Table- 4.7 Distribution of 'ever brest fed' children in different ethnic origin

Ethnic origin	Cases (missing data)	Controls (missing data)
Chinese	32 (19)	13 (37)
Vietnamese	11 (13)	5 (2)
Others	2 (0)	0 (0)

4.2.2. History of contact with diarrhoea

In this study 356 cases and 279 controls were asked for any contact history with a diarrhoea patient within the previous 2 weeks. 11 (3.1%) cases had a contact history with diarrhoea patients. None of the controls had such history.

4.2.3. Travel history within last two weeks preceding onset of diarrhoea

Travel history revealed that 38 cases had a history of travel to China within the last 2 weeks prior to admission, whereas only 4 of the controls had such a history. The difference between cases and controls was statistically significant ($p< 0.001$). However if the majority of the controls with missing data a travel history and the minority of cases with missing data had not travel, then there would be no significant difference between the two groups. The data are presented in the table- 4.8

Table-4.8 Travel history of the cases and controls

Travel history	Cases (%) (n=355)	Controls (%) (n=280)
Present	38 (10.7)	4 (1.4)
Absent	317 (89.3)	276 (98.6)
(Missing data	33	26)

4.2.4. Source of drinking water

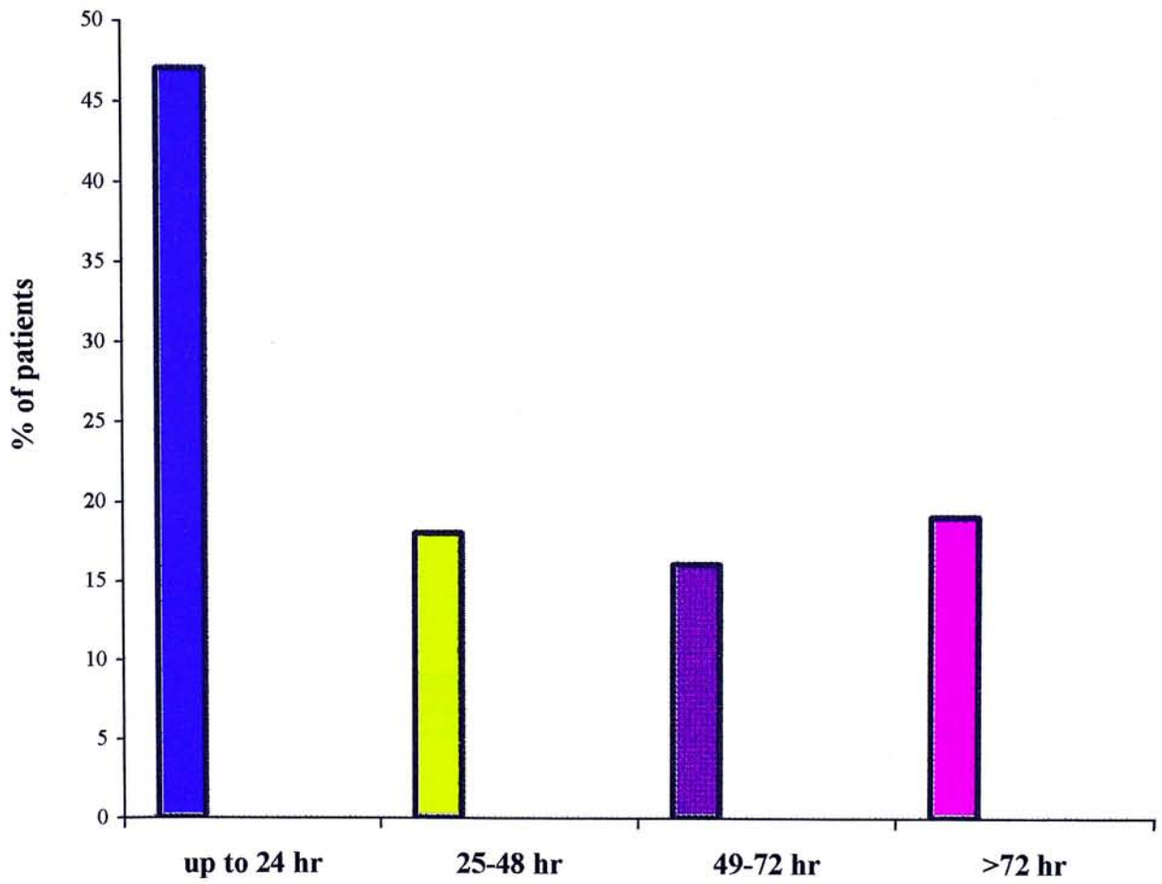
Safe water supply has been provided by the government in most of the areas of Hong Kong. Furthermore, the people were found to be more conscious about the hazards of drinking polluted water. In this study, we collected data from 300 cases and 200 controls from an enquiry about the source of drinking water in their house and whether it was taken unboiled or after boiling. For all cases and controls the water source was from the municipality piped tap water. Moreover, all but one families stated that they used boiled tap water for drinking.

4.3. CLINICAL FEATURES

4.3.1. Duration of diarrhoea at the time of admission

A significant number (44.6%) of the cases presented with a history of diarrhoea for 24 hours or less. Although few cases came to hospital after 5-6 days of diarrhoea, the number of cases who presented with a history of diarrhoea for more than 72 hours were 19.3%. These results are shown in figure-4.2

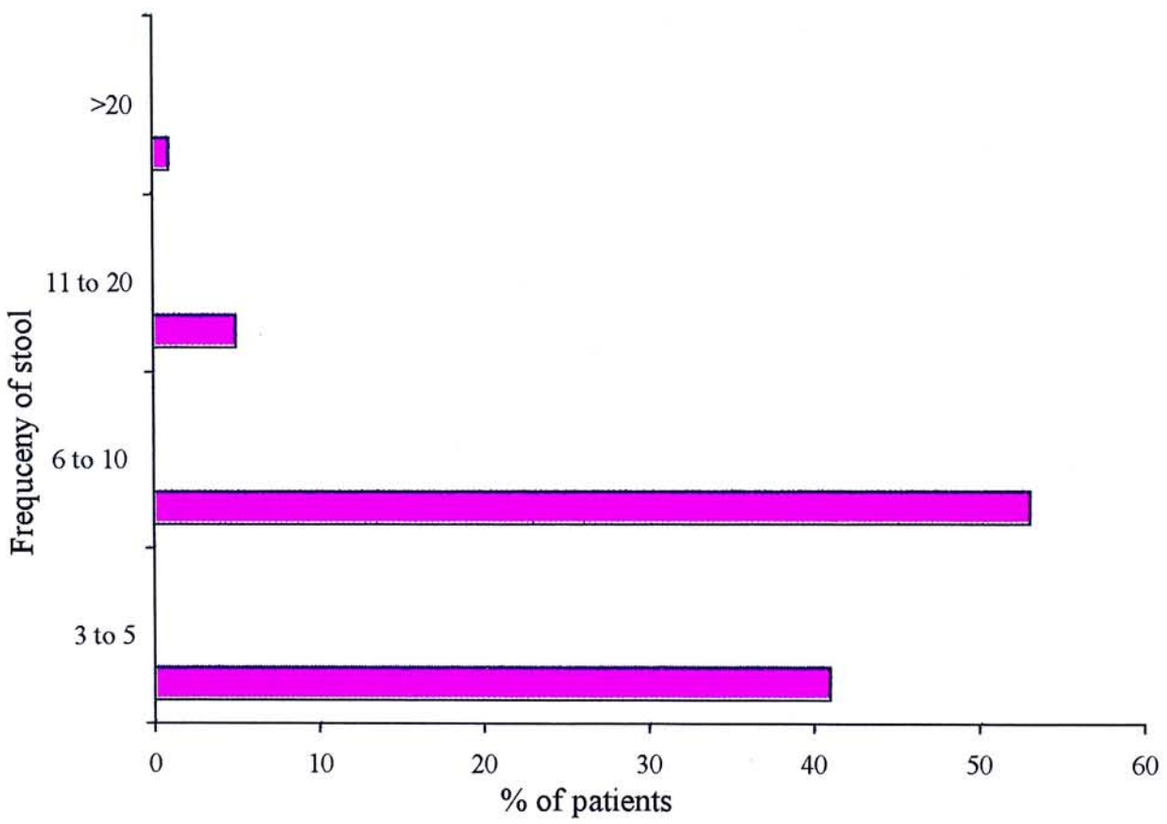
Figure- 4.2 Duration of diarrhoea before admission



4.3.2. Frequency of stool

This was the number of stools in the 24 hours before admission. Most of the patients had a history of diarrhoea for 6-10 times in the last 24 hours before admission and only 1 patient had a frequency of diarrhoea more than 20 times a day. These results are shown in figure-4.3

Figure- 4.3 Frequency of stool before the day of admission



4.3.3. Nature and contents of stool

For the majority of the cases, stool was watery and frequently it was mixed with mucous. 5 patients passed stool containing gross blood. Contents of stool are presented in the table- 4.9

Table-4.9 Contents of stool

Contents of stool	Cases (%) (n=388)
Water	159 (41.0)
Water + Mucus	109 (28.1)
Water + Blood	6 (1.5)
Water + Mucus + Blood	42 (10.8)
Mucus	36 (9.3)
Mucus + Blood	31 (8.0)
Blood	5 (1.3)

4.3.4. Condition of the perineum

The condition of the infant's perineum can give an impression of the duration of diarrhoea. In this study, these data were collected from the medical records. Over 32% of the cases had a mild erosion over the perineum. In contrast, all but two of control subjects had normal perineum. Condition of the perineum are shown in the table- 4.10

Table-4.10 showing the condition of the perineum

Condition of perineum	Cases (%) (n=365)	Controls (%) (n=289)
Healthy	243 (66.5)	287 (99.4)
Mild erosion	117 (32.1)	1 (0.3)
Excoriation	5 (1.4)	1 (0.3)
(Missing data	23	17)

4.3.5. Duration of vomiting at the time of admission

Nearly 60% of the cases and over 20% of the controls had associated vomiting. Duration of vomiting before admission are shown in the table- 4.11

Table-4.11 Duration of vomiting

Duration of Vomiting	Cases (%) (n=388)	Controls (%) (n=206)
up to 24 hours	137 (35.3)	48 (15.7)
25- 48 hours	39 (10.1)	6 (2.0)
49- 72 hours	33 (8.5)	5 (1.6)
>72 hours	19 (4.9)	5 (1.6)
No vomiting	160 (41.2)	242 (79.1)

4.3.6. Frequency of vomiting

This is the number of vomits in last 24 hours before admission. Of those patients with vomiting. 28.3% of the cases and over 13.1% of controls had a frequency of vomiting from 1-3 times. Only 9 cases had vomiting more than 10 times during the last 24 hours prior to admission. Table- 4.12 shows these results.

Table-4.12 Frequency of vomiting

Frequency of vomiting	Cases (%) (n=384)	Controls (%) (n=306)
Any vomiting	224 (58.3)	64 (20.9)
1-3 times	109 (28.3)	40 (13.1)
4-6 times	63 (16.4)	12 (3.9)
7-10 times	43 (11.2)	12 (3.9)
>10 times	9 (2.4)	0
No vomiting	160 (41.7)	242 (79.1)
(Missing data	4	0)

4.3.7. Level of dehydration in cases

Most of the cases had no signs of dehydration at the time of admission. Just under one quarter of the diarrhoea patients presented with some dehydration but no subject (cases or controls) were severely dehydrated. The dehydration status of the patients are listed in a table- 4.13

Table-4.13 Level of dehydration

Level of hydration	Cases (%) (n=388)	Controls (%) (n=293)
No dehydration (≡ <5% dehydration)	302 (77.8)	287 (98.0)
Some dehydration (≡5-9% dehydration)	86 (22.2)	6 (2.0)
(Missing data	0	13)

4.3.8. Urine output during illness

Details of urine output in the previous 24 hours were obtained by interview or from the medical records. Although none of the patients experience a cessation of urine output for 12 hours or more, 88 cases and 17 controls had evidence of reduced urine output during the illness. Data of urinary output are shown in table-4.14

Table-4.14 Urine output during the illness

Urine output	Cases (%) (n=358)	Controls (%) (n=276)
Normal	270 (75.4)	259 (93.8)
Reduced	88 (24.6)	17 (6.2)
(Missing data	30	30)

4.3.9. Fever associated with illness

Over 60% of the cases and 70% of the controls had associated fever (rectal temperature = 37.5^{0}C or more). Data are presented in the table-4.15

Table-4.15 On admission rectal temperature of the patients

Temperature	Cases (%) (n=385)	Controls (%) (n=293)
<37.5^0 C	134 (38.8)	87 (29.7)
37.5^0 C- 39.0^0 C	199 (51.7)	175 (59.7)
>39.0^{0}C	52 (13.5)	31 (10.6)
(Missing data	3	13)

The diagnosis of the control subjects were also noted from the medical records. Data were available for 297 controls, these results are shown in table- 4.16.

Table-4.16 Diagnosis of the controls subjects

Diagnosis	Controls (%) (n=297)
Asthma/ Wheezy bronchitis	64 (21.5)
Acute bronchiolitis	52 (17.5)
Febrile convulsion	42 (14.1)
Upper respiratory tract infection	24 (8.1)
Viral illness	22 (7.4)
Epilepsy	14 (4.7)
Croup or shortness of breath	10 (3.4)
Fever alone	10 (3.4)
Repeated vomiting/ gastritis	12 (4.0)
Chest infections	8 (2.7)
Urinary tract infection (UTI)	6 (2.0)
Convulsion	6 (2.0)
Neonatal jaundice	5 (1.7)
Tonsillitis	3 (1.0)
Liver diseases	3 (1.0)
Miscellaneous (NAI, ITP, HSP, Nephrotic Syndrome etc.)	16 (5.4)
(Missing data	9)

4.4. HISTORY OF HOME- MANAGEMENT

4.4.1. Main food taken at home during illness

Food taken by the patients during illness at home are listed in the table- 4.17

Table-4.17 Main food taken at home during the illness

Main intake during illness	Cases (%) (n=374)	Controls (%) (n=287)
Formula	279 (74.6)	186 (64.8)
Breast milk	10 (2.7)	4 (1.4)
Cow's milk	6 (1.6)	5 (1.7)
Other / Normal diet	79 (21.1)	92 (32.1)
(Missing data	14	19)

4.4.2. Supplementary fluid taken at home during illness

In the present study, a question was asked to the accompanying person about what type of fluid was given to the patient during illness. Over 82% of the cases and nearly 99% of controls drunk water as supplementary fluid. Only 15.5% of the cases chose ORS (Oral rehydration solution) for rehydration. Data are presented in the table- 4.18

Table-4.18 Supplementary fluid intake during illness

Supplementary fluid intake	Cases (%) (n=355)	Controls (%) (n=274)
Water	292 (82.3)	271 (98.9)
ORS (Oral Rehydration Solution)	55 (15.5)	1 (0.4)
Fruit juice	8 (2.3)	2 (0.7)
(Missing data	33	32)

4.4.3. Duration of hospital stay

Duration of hospital stay in cases and controls were comparable. The mean duration of hospital stay for cases and controls were 3.2 days and 3.5 days respectively. The difference between the two groups was not statistically significant ($p>0.05$). The results are shown in the table- 4.19

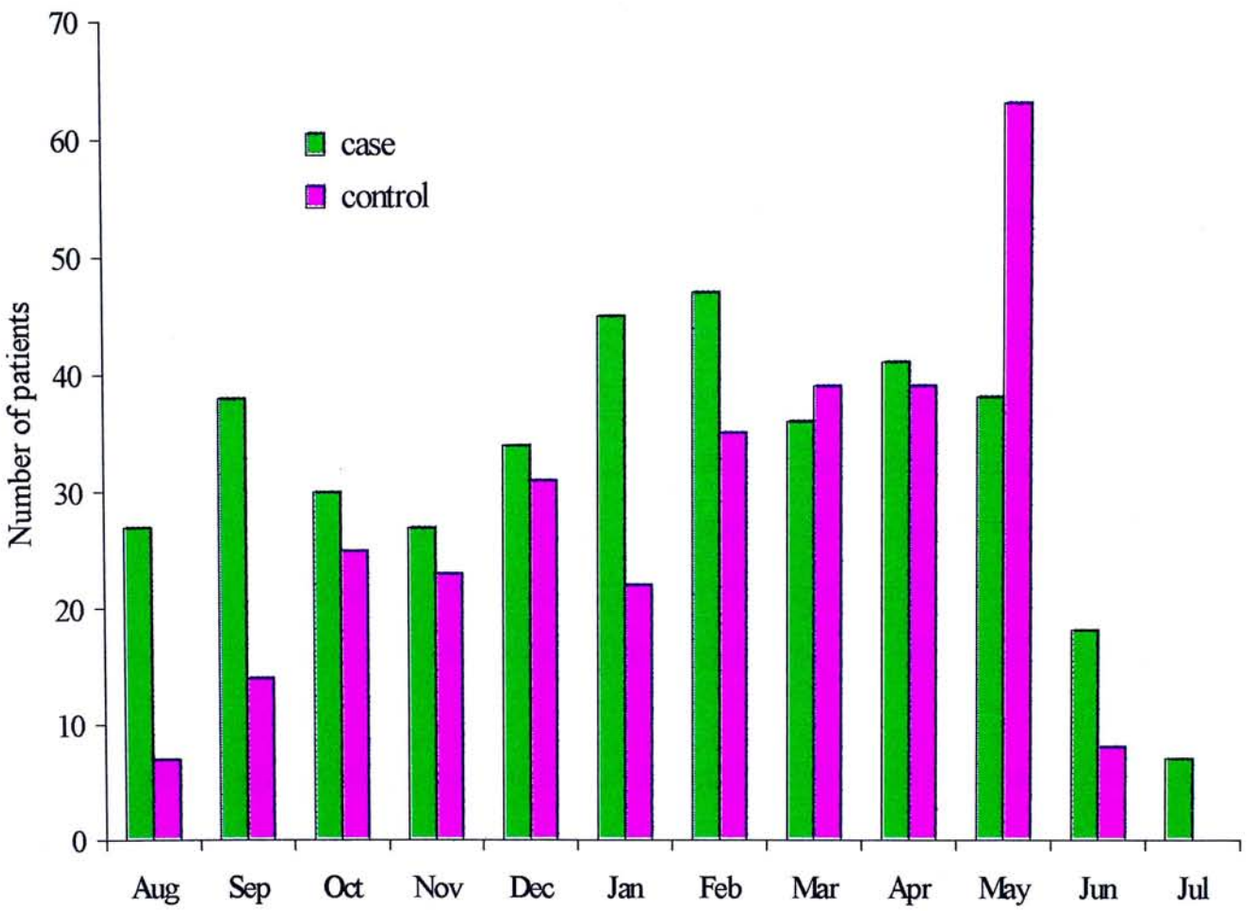
Table- 4.19 Duration of hospital stay for cases and controls

Duration of hospital stay	Cases (%)	Controls (%)
1 day	80 (20.8)	58 (19.0)
2-3 days	200 (51.9)	134 (43.9)
4-7 days	84 (21.8)	97 (31.8)
8 days or more	21 (5.5)	16 (5.3)
(Missing data	3	1)

4.4.4. Recruitment of patients in different months

The number of cases and controls recruited for the study in different months of the year is shown in figure-4.4

Figure- 4.4 Recruitment of patients in different months



4.5. RESULTS OF GENE PROBING FOR *E. COLI*

We analysed all of our 4300 saved *E. coli* colonies from a total of 430 subjects, of which 253 were cases and 177 were controls (10 picks from each patients) with 8 different probes. 3 probes were specific for EPEC, 3 probes for ETEC and 1 probe for each of EAggEC and DAEC. EHEC and EIEC were not assessed in this study. Any one of the 10 colonies positive with a probe was considered as positive for the particular patient.

The results of DNA probes analysis demonstrated that only EAF (EPEC Adherence Factor) positive EPEC cases showed a statistically significant difference with the controls ($p = 0.005$). EAF probe was positive in 10 cases and in none of the controls. Only one of these cases simultaneously possessed *eae* (*E. coli* attaching and effacing) gene. However 8 of these 10 cases had other pathogens also isolated and only 2 may have caused diarrhoea specifically by EPEC infection. *eaeA* probe was positive in equal number of cases (12/388, 3.1%) and controls (12/306, 3.9%), and thereby did not show any association with diarrhoea. *bfpA* (bundle forming pilus) did not react with any specimen.

DA (diffusely adherent) probe was used to identify the diffusely adherent *E. coli* (DAEC). DAEC has been described as the 6th category of *E. coli* in some literature [Theilman. 1994). In our study, DAEC did not seem to be associated with diarrhoea as it was positive in 21 cases and 16 controls. This result was not statistically significant ($p>0.05$).

In this study enteroaggregative *E. coli* (EAggEC) also was not associated with diarrhoea as it was positive in 9 cases and 3 controls ($p>0.05$).

Enterotoxigenic *E. coli* (ETEC) was investigated with 3 specific probes, LT, STIa and STIb. It has also been found that ETEC are not associated with childhood diarrhoea in Hong Kong. The results of gene probing are shown below in the table- 4.20

Table-4.20 Results of gene probing

Probes	No. of cases	No. of controls	P-value
EAF+ (EPEC Adherence Factor)	10	0	<0.05
eaeA+ (*E. coli* attaching and effacing)	12	12	NS
DA+ (Diffusely adherent)	21	16	NS
EAgg+ (Enteroaggregative)	9	3	NS
LT+ (Heat Labile Toxin)	7	5	NS
bfpA+ (Bundle forming pilus)	0	0	
ST Ia+ (Heat Stable Toxin)	0	0	
ST Ib+	0	0	
Total	59	36	

NS= not statistically significant.

4.6. DETAILS OF THE EAF+ EPEC CASES

9 out of the 10 EAF+ cases were Chinese. 6 of these cases were male and 4 were female. Most (8) of the patients were under 2 years (2-15 months). All patients were nutritionally healthy, mean BMI = 15.2 (range 12.0-18.0). We collected history of breast feeding from 9 patients and none of them was 'ever breast-fed'. Table- 4.21 details these 10 EAF+ cases.

Table- 4.21 Demography of the EAF+ cases

EAF+ cases	Ethnic origin	Sex	Age in months	Duration of diarrhoea (hrs)	BMI	Breast feeding	Days in hospital
Case-1	Viet	F	12.2	48	12.0	----	5
Case-2	Chi	M	12.3	24	15.2	N	4
Case-3	Chi	F	2.6	24	14.7	N	6
Case-4	Chi	M	33.2	24	16.5	N	2
Case-5	Chi	M	9.5	48	16.2	N	1
Case-6	Chi	F	84.1	48	12.0	N	2
Case-7	Chi	M	15.0	24	15.8	N	3
Case-8	Chi	M	10.5	24	16.3	N	5
Case-9	Chi	F	4.4	24	18.0	N	12
Case-10	Chi	M	2.1	24	15.0	N	2

Viet= Vietnamese, Chi= Chinese.

The average duration of diarrhoea prior to admission was 24 hours although 3 patients had diarrhoea for 48 hours. Mean frequency of stool was 6.6 which was similar to EAF-negative patients. Stool was mainly watery with or without mucus. One patient had bloody diarrhoea and one had only mucoid stool. Although 4 patients had associated vomiting, most of the patients (9) were well hydrated and none had reduced urine output. 7 patients suffered from associated fever. None had travel history .

Mean duration of hospital stay were 4.2 days (1-12 days). Out of 10 cases, 5 patients were admitted in April, 1995.

4.6.1. Associated infections in EAF+ cases

More than one pathogen had been isolated from all but 2 of 10 EAF+ EPEC cases. The table- 4.22 shows the result of different probes, stool culture and rotavirus assay, which were associated with EAF+ cases.

Table-4.22 Associated infections in EAF+ cases

EAF+	DA	eaeA	EAgg	LT	Rotavirus	Stool culture
Case-1	-	+	-	-	+	-
Case-2	-	-	-	-	-	Aeromonas-sobria and Salmonella-D
Case-3	-	-	-	-	+	-
*Case-4	-	-	-	-	-	-
Case-5	-	-	-	-	-	Salmonella-C
Case-6	+	-	-	-	-	Salmonella-D
Case-7	-	-	+	-	+	-
Case-8	-	-	-	-	-	Salmonella -E
*Case-9	-	-	-	-	-	-
Case-10	-	-	+	-	-	Salmonella-E

* infected with EAF+ EPEC only.

4.7. AETIOLOGY OF DIARRHOEA

In this study, routine culture of the stool specimens was performed for common bacteria. ELISA was performed for rotavirus and the specimens were also examined under microscope for ova and cysts. The results in the cases and controls are listed below in table-4.23

Table-4.23 Enteropathogens isolated from children with diarrhoea and controls

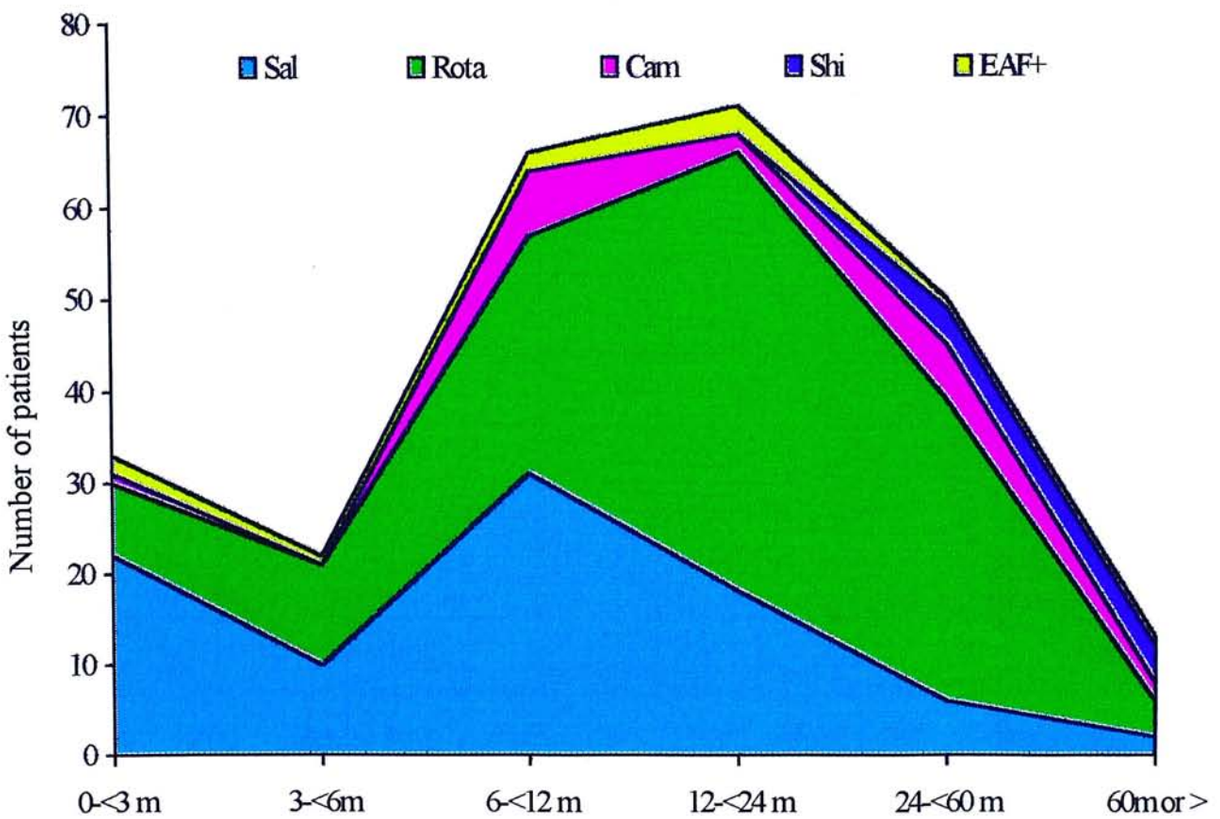
Enteropathogens	Cases (n=388)	Controls (n=306)
Rotavirus	130 (34.6%)	Not done
Bacterial pathogens	116 (30%)	17 (5.6%)*
Salmonella	89 (23.3%)	13 (4.2%)*
Group B (including S. typhimurium)	42 (10.9%)	3 (1.0%)*
S. typhimurium	12 (3.1%)	1 (0.3%)
Group-C	19 (4.9%)	3 (1%)*
Group-D	7 (1.8%)	0
Group-E	21 (5.4%)	7 (2.3%)*
Campylobacter	18 (4.7%)	2 (0.7%)*
Shigella	8 (2.1%)	0
Aeromonas	1 (0.3%)	2 (0.7%)
Giardia lamblia (cyst)	1 (0.3%)	0

* P<0.0001

4.7.1. Age distribution

Age distribution associated with the isolated pathogens are shown in figure-4.5. *Salmonella* infected young infants under 6 months more than the other pathogens, whereas all the *Shigellosis* cases were above 2 years of age.

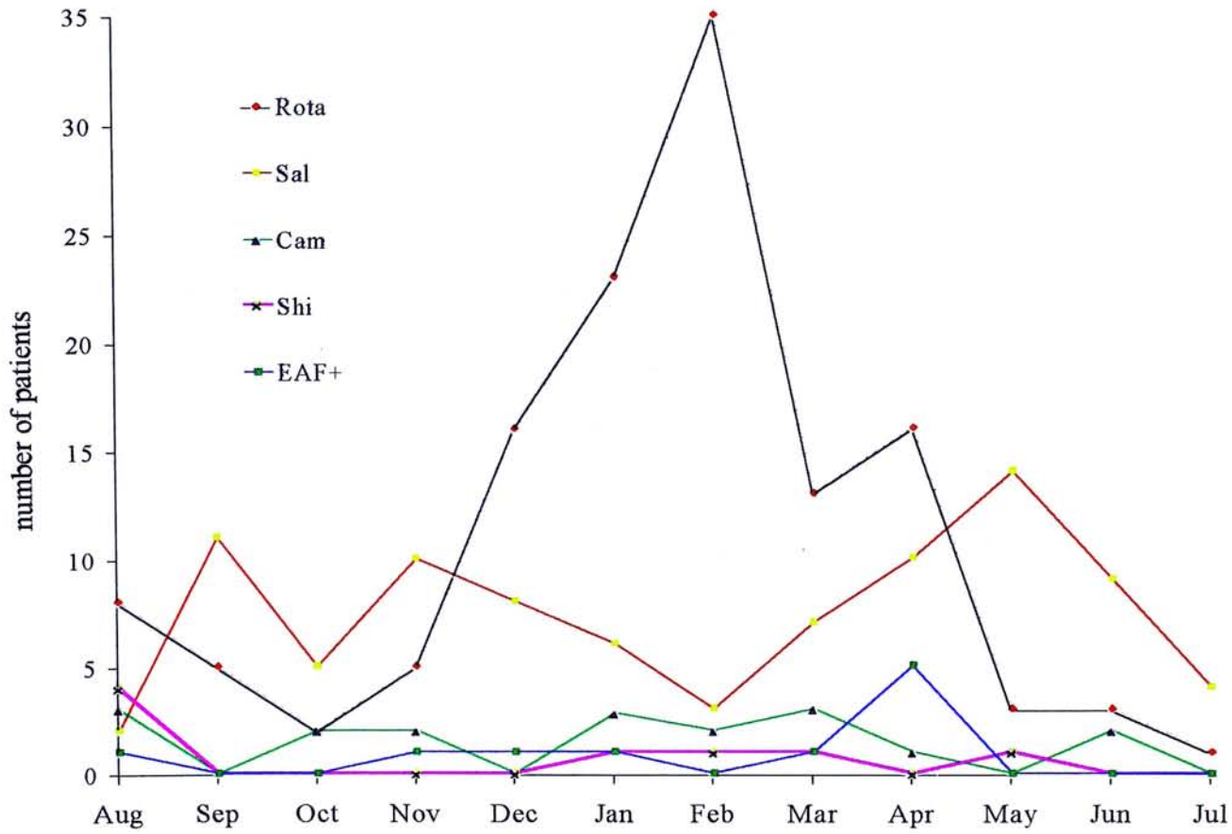
Figure- 4.5 Age distribution of isolated enteropathogens



4.7.2. Seasonal distribution

Seasonal variation of different pathogens isolated are presented in figure- 4.6. A definitive peak has been shown for rotavirus in the months of January and February. *Salmonella* has been isolated as an endemic cause of diarrhoea without any obvious peak. 5 of the 10 EAF+ EPEC cases were isolated in April.

Figure- 4.6 Seasonal distribution of isolated enteropathogens



4.7.3. Clinical features

Major clinical findings resulted from different infections by commonly isolated pathogens are shown in the tables- 4.24A and B. Vomiting was more commonly associated with rotaviral diarrhoea and fever was noted in all cases of *Shigella* and nearly 90% of Campylobacter infection. Stool caused by rotaviral diarrhoea was mostly watery, whereas other groups had blood stained stool in and around 50% cases except the EAF+ cases. Some dehydration was present in average one quarter of cases in all but EAF+ groups.

Table- 4.24A Clinical features of commonly isolated enteropathogens

Pathogen (n)	Vomiting	Fever	Some dehydration
Rotavirus (130)	78.2%	70.7%	28.5%
Salmonella (89)	43.3%	68.5%	17.6%
Campylobacter (18)	38.8%	88.8%	27.8%
Shigella (8)	75%	100%	25%
EAF+ (10)	40%	70%	10%

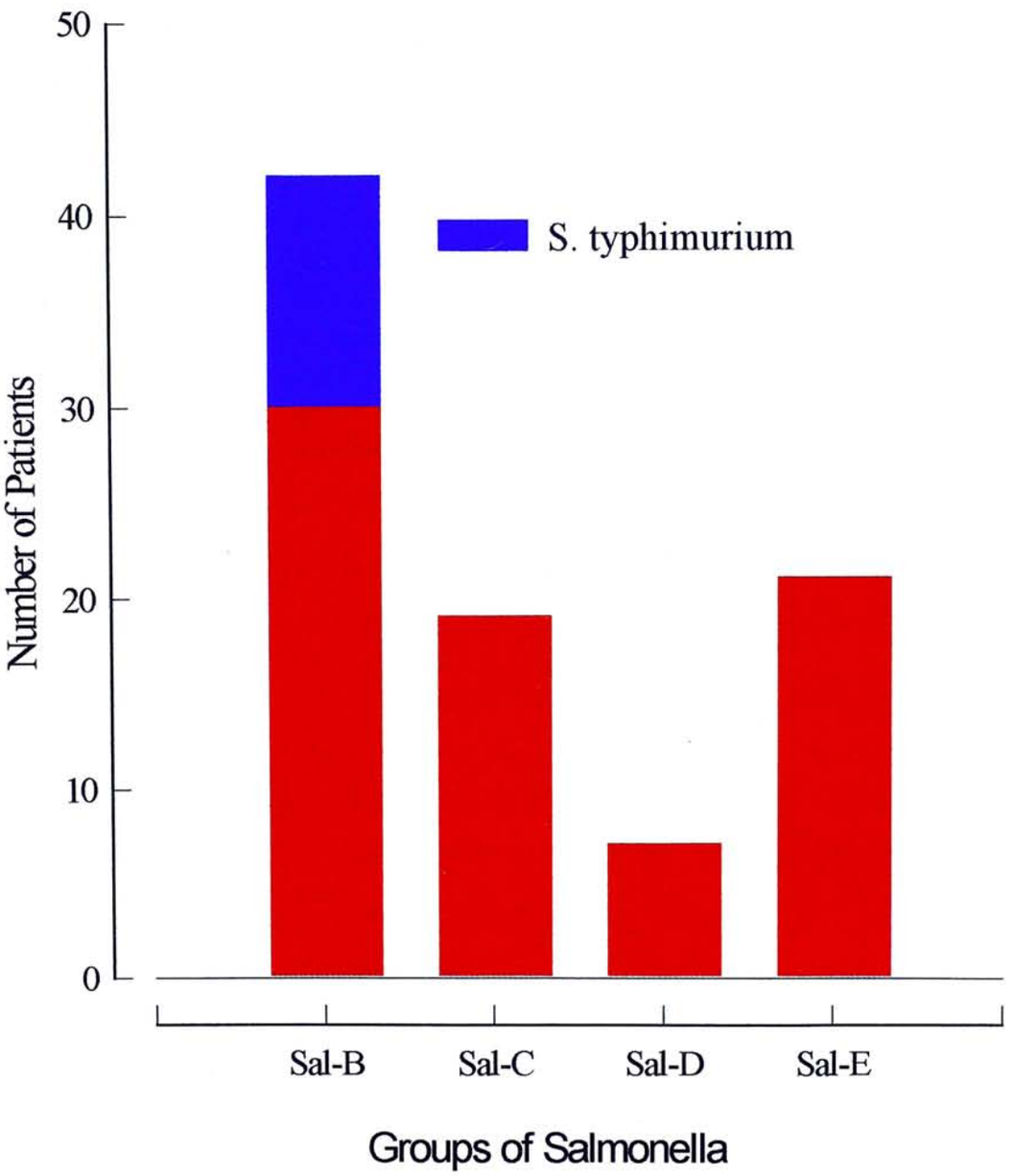
Table-4.24B Nature and frequency of stool

Pathogen (n)	Water in stool	Mucus in stool	Blood stained stool	Stool frequency
Rotavirus (130)	86%	50%	3%	6.9
Salmonella (89)	68.1%	72.5%	49.5%	7.0
Campylobacter (18)	77.8%	83.3%	55.6%	7.1
Shigella (8)	87.5%	62.5%	62.5%	11.5
EAF+ (10)	90%	60%	10%	6.6

4.7.4. Different groups of *Salmonella*

Prevalence of different groups of *Salmonella* in cases are shown in figure-4.7

Figure 4.7 Different groups of Salmonella isolated



4.7.5. Dual infection among enteropathogens isolated

The cases were infected by two pathogens simultaneously are shown in the table- 4.25

Table- 4.25 showing dual infection among enteropathogens

Bacteria	Sal-D	Sal-E	Sal-T	Shigella	Rotavirus
Aeromonas-sobria	1	0	0	0	0
Campylobacter	0	0	0	1	3
Salmonella-B	0	0	1	0	1
Salmonella-C	0	1	0	0	1
Salmonella-D	0	0	0	0	1
Salmonella-T	0	0	0	0	2
Salmonella-E	0	0	0	0	4

Chapter-5

DISCUSSION

The main objectives of this study were to determine the epidemiology and aetiology of diarrhoea in hospitalised children in Hong Kong with a particular emphasis on the prevalence of EPEC. A hospital-based prospective case-control study was designed to define the role of EPEC in diarrhoea in Hong Kong children, to examine the morbidity of diarrhoea in this group of children in relation to aetiological factors, and other factors associated with the caring of the children. The vital signs (pulse, respiration, temperature) and other important clinical features were recorded at the time of admission. In addition, the epidemiology of specific aetiologic agents causing diarrhoea in this group of children were studied. This was compared with a control group admitted concurrently in the same hospital but without diarrhoea. Detailed history were obtained on admission and used in the assessment of severity of diarrhoea in relation to various risk factors associated with the disease and aetiology.

The stool specimen collected from both the cases and controls were investigated in the laboratory for bacteria, virus and parasites with the facilities available locally. Gene probing was then undertaken to identify various categories of *E. coli*.

Variables requiring information from interview such as history of breast feeding and travel history will have been affected by limitations related to data collection as described in the Methods section. Other variables based on laboratory data or consistently recorded in the medical record would not be so affected.

5.1. RISK FACTORS ASSOCIATED WITH DIARRHOEA

5.1.1. Age and sex of the patients

Diarrhoea tends to be a health problem which usually affects children. In particular, EPEC infection is more commonly identified in young infants under the age of two years [Edelman and Levine. 1983, Robins-Browne. 1987]. In this study children (up to 15 years) were eligible for recruitment. So the result of this study may not be comparable with some other studies, where younger children of selected age group were studied. However, it was found that nearly 80% of the cases with diarrhoea were under 2 years. This data suggested that like the other parts of the world, children of younger age group are also more vulnerable to diarrhoeal illness in Hong Kong. In this study, a male predominance has been observed in both cases and controls.

5.1.2. Nutritional status

In developing countries, an increased risk of diarrhoea is demonstrated for those children with poor nutritional status and conversely, a high incidence of diarrhoeal disease may precipitate undernutrition. Hence, malnutrition and diarrhoea can form a vicious cycle. Furthermore, improvements in the nutritional status have been identified as one of the more important strategies for controlling diarrhoeal diseases in the developing nations by the World Health Organisation. But nutritional status in Hong Kong children have been assessed by a series of studies conducted by Leung and colleagues and as a whole a good nutritional status has been demonstrated among these children. It should be noted that at present nutritional status of Hong Kong children have been changed a lot with the rapid socioeconomic development since 1960s [Leung and Lui, 1990; Leung *et al.* 1992]. It should also be emphasised that malnutrition as seen in the developing world (marusmus/ kwashiorkor) is extremely uncommon in Hong Kong. Nutritionally healthy Hong Kong children are found to be shorter and lighter than that of Caucasian children, but this difference is due to genetic factors [Leung and Lui, 1989].

5.1.3. Breast feeding

Breast milk has a definitive role in prevention of many diseases in young children. Diarrhoea caused by different pathogens including EPEC can also be prevented by breast feeding [Popkin *et al.* 1990, Fang. 1993] and the protective effect of breast feeding on diarrhoeal disease is a complex interaction of several factors. The immunological and antimicrobial properties of breast milk is one of the major factors considered. The passive transfer of antimicrobial secretory antibodies through breast milk have been most actively studied [Welsh *et al*, 1979]. Many authors have reported the presence of antibacterial antibodies in breast milk and these studies were reviewed by Goldman and Smith in 1973. It was also described in that report that antibacterial antibody was particularly focused on anti-*E. coli* IgA , because of the common involvement of *E. coli* in infantile diarrhoea. Breast milk is thought to act by inhibiting the adhesion of EPEC to enterocytes by providing secretory IgA or an oligosaccharide-enriched fraction purified from colostrum and breast-milk [Cravioto *et al.* 1991]. Another mechanism by which breast feeding may be protective against diarrhoeal disease is the reduced probability of environmental contamination of oral intake. Those infants taking milk formula are at risk of contamination of the bottle, water or the milk itself and those who are taking solid food (especially at the weaning period) are also vulnerable to be infected by the pathogens contained in the food material. These risks may be applicable particularly to bacterial pathogens that are found in the water used in the preparation of milk or food, and for cleaning utensils. In Hong Kong breast feeding rate is very low. A report from the survey, carried out in 1995 by the Baby Friendly Hospital Initiative, Hong Kong Association, the rate of ever breast feeding on discharge from hospitals was 32.4%. We had some limitations in obtaining the exact data of breast feeding in this study, as in a number of patients it was not possible to get the information of early breast feeding directly from the mother. When the information was not recorded in the notes also, it appeared as missing data. Despite this limitation it is apparant that in the present study only a very few cases and controls were breast feeding during the time of admission. The term 'breast feeding' was used in this study to refer 'ever breast-fed' and the data were higher in the cases than the controls. It should be noted that these subjects were not exclusively breast-fed. So the optimal efficacy of the protective role of exclusive breast feeding may not be manifested in

these children. Furthermore, it could be due to the fact that the hygiene and sanitation are satisfactory in Hong Kong, so the anti-infectious protective role of breast milk is less evident here [Toledo *et al.* 1983]. However, breast feeding in Hong Kong may reflect lower socioeconomic status. Poor people like recent immigrants from mainland China or Vietnamese refugees residing in detention camps are at greater risk of gastroenteritis, although they might have a higher breast feeding rate. In this study, a relatively higher rate of breast feeding has been observed among the Vietnamese children than the Chinese children. As a whole, a low incidence of breast feeding has been documented in Hong Kong and this may be one of the contributing factors to diarrhoeal illness especially in younger infants.

5.1.4. Travelling

Travelling has been identified to be a risk factor for diarrhoea in this study. A significantly higher number of cases than controls had a history of travel within the last two weeks prior to admission to the hospital and in most of the cases it was to mainland China. This increased incidence of diarrhoea may be due to contact with patients suffering from diarrhoea or with asymptomatic carriers of the disease. Transmission of diarrhoeal pathogen through polluted drinking water or contaminated food might also be responsible. However because of missing data we can not confidently say exactly how important this factor is.

5.2. SEVERITY OF DIARRHOEA IN HONG KONG

The severity of diarrhoeal illness can be determined by the general condition and also based on the vital signs on admission, status of hydration, nutrition, and finally recovery of the patients from the disease. In addition to these, duration, frequency and contents of stool should also be considered. Our study was a hospital based study and we would expect the more severe cases of diarrhoea in this study. The milder cases usually do not require admission and many patients are treated by the general practitioners and community health workers. Despite the hospitalised children being representative of the more severe cases of diarrhoea of the community, in our study it appears to be a mild disease in most of the patients in comparison to the diarrhoeal patients of the developing countries. This may be due to the better hygiene, good treatment facilities and better

nutrition of Hong Kong children. Vomiting was associated with diarrhoea in some cases and dehydration was noted in 22% of total patients, where none had severe dehydration. Fever was another common symptom of these children, but it was very difficult to find out the exact relation of fever with diarrhoea in these cases as the other possible causes of fever were not excluded. The majority of patients had loose or watery stool, with or without blood staining and a history of bowel opening on average 7-8 times during last 24 hours before admission.

Only a few cases presented with gross blood in the stool and a serious systemic infection may occur in young infants presented with blood stained stool and high fever. Such infants should be carefully monitored and may require antibiotic therapy.

5.3. MOLECULAR EPIDEMIOLOGY OF EPEC IN HONG KONG

For the detection of EPEC, 3 specific probes (EAF, *eae*A and *bfp*A) were used. Only the result of EAF probe demonstrated statistically significant difference between the cases and controls as shown in the table-4.19. 12 cases and equal number of controls were positive with *eaeA* probe, thereby showing no association with diarrhoea. Furthermore, none of the colonies was positive with *bfpA* probe. It could be concluded that EAF+ EPEC strains were associated with diarrhoea only in 10 of 388 (2.6%) children.

Poor correlation of the results from different probes for the same patients are not new. Recent advances in the understanding of the pathogenesis of EPEC and development of highly sensitive and specific probes for detection of different categories of *E. coli* has dramatically altered the situation making it comparatively easier to undertake comprehensive studies. However, the correlation among the results of different probes or techniques for the identification of EPEC are still not well established and these variations remain an unresolved problem with EPEC, and to some extent to *E. coli* in general.

5.3.1. EAF probe

EPEC was defined as diarrhoeagenic *E. coli* belonging to serogroups epidemiologically incriminated as pathogens, but their pathogenic mechanisms have not been proven to be related either to heat-labile toxins (LT); heat-stable toxins (ST) or to *Shigella*-like invasiveness [Edelman and Levine, 1983]. Serogrouping was used to be the only method of diagnosis of EPEC. In 1979, Cravioto *et al* demonstrated that unlike typical *E. coli*

strains, 80% of EPEC strains adhered to HEp-2 cells. This was associated with localised adherence (LA) pattern, which was characteristically in tight clusters or microcolonies [Nataro *et al.* 1987]. This finding resulted in HEp-2 cell assay being used as a new diagnostic tool for EPEC. Later on, this characteristic adherence of EPEC to culture cells was found to be encoded on a 50 to 60 MDa plasmid, referred to as the EPEC adherence factor (EAF) plasmid [Baldini *et al.* 1983]. A 1-kbp DNA probe derived from the EAF plasmid of EPEC E2348/69 (O127:H6), termed EAF probe [Nataro *et al.* 1985b], has been used extensively in epidemiological studies as a highly sensitive method to identify and characterise EPEC strains that show LA to culture cells [Echeverria *et al.* 1987, 1992; Nataro *et al.* 1985a, 1985b; Chatkaemorakot *et al.* 1987]. This well established and highly sensitive probe could be considered as a standard method for the identification of classical EPEC serogroups.

5.3.2. EAF and EPEC virulence

EAF probe has been shown to be 99 to 100% sensitive and specific in predicting LA to HEp-2 or HeLa cells [Nataro *et al.* 1985a, 1985b; Gomes *et al.* 1989]. *E. coli* strains demonstrated to be EPEC by both EAF probe and serotype, have been shown to be highly associated with diarrhoea. Such good correlation has not been found between EAF-negative EPEC serotypes and diarrhoea [Taylor *et al.* 1993]. Furthermore, an association has been demonstrated between the ability of EPEC to show LA and positivity for EAF probe [Nataro *et al.* 1985a]. In a study by Levine *et al.* (1985) EAF-negative EPEC resulted in significantly fewer cases of diarrhoea compared to the wild type EAF + strain. This indicated that the EAF plasmid is required for expression of the full virulence of EPEC.

Discrepancies have always been noted between serogrouping and EAF probe analysis as only half [Echeverria *et al.* 1989] or one-third [Sunthadvanich *et al.* 1990] EAF+ strains were actually found to be EPEC serotypes. Furthermore, Cravioto *et al.* (1991a) have reported that half the strains with localised adherence were not EPEC serotypes nor did they hybridize with an EAF probe. This has been attributed to the fact that certain EPEC serotypes were non-adherent to HEp-2 or HeLa cells. As EAF probe is only positive with the EPEC showing LA to these cell lines, the non-adherent serotypes would therefore remain undetected [Begaud *et al.* 1993]. As a result of further

observation, it was noted that not all EPEC strains were EAF positive and high rate of plasmid loss (66%) seen in human volunteers infected with EAF+ EPEC. If the same thing occurs in infants with EPEC infections, then there is a high probability that *E. coli* strains isolated from stool will be EAF-negative, even though the original pathogen was EAF positive [Levine *et al.* 1985]. On the basis of the EAF probe reaction, EAF+ EPEC serogroups in which EAF was almost always found have been designated as class-I EPEC and serogroups in which EAF was rarely found have been designated as class II. Both class I (EAF+) and class II (EAF-negative) were associated with diarrhoeal disease [Nataro *et al.* 1985b], but class II were pathogenic by a mechanism not involving HEp-2 cell adhesiveness [Levine *et al.* 1985]. Until the discovery of a new gene probe, the commonly used approaches for the identification of true EPEC strains were serogrouping, a localised phenotype in the HEp-2 cell assay and positivity with the EAF probe.

5.3.3. *eaeA* probe

The *eae* gene has been identified as being essential for the characteristic intimate attachment of EPEC to epithelial cells [Jerse *et al.* 1990]. This gene is chromosomally encoded in all EPEC and EHEC strains tested as well as in a rabbit diarrhoeagenic *E. coli* (RDEC-1), *Citrobacter furundi* and *Hafman alvei* [Cantey *et al.* 1977, Donnenberg and Kaper. 1992, Francis *et al.* 1986, Tzipori *et al.* 1986]. The *eaeA* probe was developed from this gene and it was shown to be highly sensitive and specific in detecting *E. coli* of EPEC serogroups demonstrating AE activity [Jerse *et al.* 1991b]. However, this probe has the disadvantage of not being able to distinguish between EPEC and EHEC strains.

Not only this, there are also some lack of correlation between *eaeA* and EAF probe. It has been demonstrated that *E. coli* of EPEC serogroups that hybridized with *eaeA* probe and produced A/E activity did not hybridize with EAF probe [Jerse *et al.* 1991b; Schimidt *et al.* 1994; Albert *et al.* 1994]. An EPEC strain cured of the EAF plasmid was shown to have reduced ability to form AE lesions and to cause actin aggregation in tissue culture compared to the EAF-expressing parent [Knutton *et al.* 1987, 1989a]. It indicates that the EAF plasmid of EPEC strain may have a regulatory role in the production of AE activity [Jerse *et al.* 1991a].

5.3.4. FAS test

Fluorescence Actin Staining (FAS) test was described on the basis of accumulation of actin in the apical cytoplasm of the epithelial cells beneath the attached bacteria, which was characteristic of AE membrane lesion. This test was shown as a simple and highly sensitive diagnostic test for EPEC and EHEC which cause AE activity [Knutton *et al.* 1989a]. EPEC and EHEC, both produced an AE lesion in the brush border of microvillous membrane by adhering to the intestinal mucosa. This AE lesion was characterised by localised destruction of microvilli and intimate attachment of bacteria to the apical enterocyte membrane. In both cases, dense concentrations of microfilaments were present in the apical cytoplasm beneath the attached bacteria. Using a fluorescein-labelled phallotoxin, it has been shown that these microfilaments were composed of actin. Cells infected with EPEC and EHEC strains which produce AE lesion all exhibited spots of fluorescence which corresponded in size and position with each adherent bacterium. However, cells infected with other adherent *E. coli* strains known to produce the AE lesion did not show this striking pattern of fluorescence and were indistinguishable from uninfected control cells.

5.3.5. *bfpA* probe

It has been described more recently that EPEC express an inducible bundle forming pilus (BFP) associated with the presence of EAF plasmid and LA on HEp-2 cells. The cloned structural gene *(bfpA)* encoding BFP was found to be specific for EPEC, as the homologous sequence were found only in EPEC and not in other enteropathogens. Among EPEC strains with LA, *bfpA* and EAF probes hybridized with 99% and 96% of the strains respectively. It may also be positive in some EAggEC [Giron *et al.* 1993a].

5.3.6. Comparison and contrast among the probes

Although much knowledge have been acquired over the time and a significant number of gene probes have been described as the specific methods for the isolation of EPEC, the practical experiences after using these probes for the diagnosis of EPEC were not as good as it was expected. As the number of identifying methods are increasing, the possibilities for controversy are also increasing, as none of *eaeA, bfpA* and FAS test are absolutely specific for EPEC.

It has been shown in a study that all EPEC and non-EPEC isolates that hybridized with EAF probe were FAS+, but not all FAS+ strains hybridized with the EAF probe. EAF+ EPEC were significantly associated with diarrhoea; neither EAF+ non-EPEC nor EAF-negative EPEC were isolated from cases more often than controls. Even EAF-negative O55 EPEC isolates that caused an AE lesion as detected by the FAS test were not associated with diarrhoea [Echeverria *et al.* 1991].

Using colony hybridization, screening was done by Schmidt *et al.* (1994) for the presence of *E. coli* carrying *eae* gene among 237 samples from patients with diarrhoea and 237 samples from age matched controls. Individual colony harbouring *eae* gene could be recovered from 7 (2.9%) cases as well as 6 (2.5%) control stools. All the samples were probed for Shiga-like toxin (SLT) and EAF to evaluate whether testing for *eae* identified all EHEC and class-I EPEC. Of the 7 patient samples harbouring *E. coli* with *eae*, 4 had *E. coli* with *eae* and SLT genes, and 2 had *E. coli* with *eae* and EAF sequences. Of the control samples, none of the *E. coli* strains including the 6 samples containing *eae* positive strains, possessed EAF or SLT sequences [Schmidt *et al.* 1994]. In the same study, it was also shown that the *eaeA* probe recognised all 26 class I EPEC and 75 of 82 EHEC strains. It has therefore been suggested that colony hybridization using the *eaeA* probes appeared to be of little diagnostic value since the incidence of *eaeA* probe positivity was almost identical in patients with diarrhoea as well as in controls. Similarly, from the findings of our study, equal number of cases and controls were positive for *eaeA* probe. EPEC serogroups from seven control children were EAF-negative but *eaeA*+, was reported from another study [Albert *et al.* 1994]. Not so many reports are available where *eaeA* probe has been used. The specific role of this probe in EPEC diagnosis needs further evaluation.

Another study was conducted utilising FAS test and it was found that over 50% of EAF probe-negative strains were FAS-positive (13 of 23) [Knutton *et al.* 1991]. The *eae* of the AEEC (attaching-effacing *E. coli*) is chromosomally encoded, and it was demonstrated that the EAF plasmid, in addition to enabling initial colonisation, may provide tissue specificity for human epithelial cells. These findings confirmed the existence of both plasmid encoded and chromosomally encoded virulence factors in AEEC. Although it was known that the EAF plasmid increased the virulence of an EPEC strain

[Knutton *et al.* 1987; Levine *et al.* 1985; Tzipori *et al.* 1989], the function of the plasmid sequences detected by the EAF probe has yet to be elucidated. The EAF probe sequences are highly conserved among those AEEC that demonstrate LA to HEp-2 cells [Nataro *et al.* 1987], but it is not known whether these sequences are part of DNA that encodes the actual adhesin. In contrast, the chromosomal *eaeA* gene is present in all AEEC and has been clearly shown to encode a membrane protein involved in the production of the AE lesion. The role and importance of both chromosomal and plasmid-encoded elements in virulence needs to be addressed, as this information has implications in understanding the pathogenesis of EPEC as well as in the definition and diagnosis of the pathogen [Jerse *et al.* 1991b].

Although our knowledge of EPEC pathogenesis has significantly increased in recent years, the relative contribution of plasmid-encoded virulence factors versus chromosomally encoded factors remains unclear. Studies that compared the isolation of EAF-positive *E. coli* of EPEC serogroups from cases versus controls support both the validity of the EAF probe in identifying EPEC isolates and the association of HEp-2 adherence with virulence [Gomes *et al.* 1989, Levine *et al.* 1988]. Until the epidemiological significance of EAF probe-negative *E. coli* that produces AE activity in vitro is known. However, it is perhaps advisable to screen potential EPEC isolates for AE activity as well as for EAF probe positivity [Knutton *et al.* 1991].

5.3.7. Probes in the present study

In our study, we followed the idea given by Knutton *et al.* (1991) for analysis of the specimens and used EAF, *eaeA* and *bfpA* probes. The results demonstrated only a statistically significant difference between the cases and controls with EAF probe, but *eaeA* probe did not show any diagnostic value. Although *bfpA* probe has been described as being highly (99%) sensitive test for EAF plasmid containing EPEC [Giron *et al.* 1993a], in our study, none of the 10 EAF-positive cases reacted with this probe. This *bfpA* probe has been used in a very limited number of studies since it was described. We found only one published report (in 1994) up to date, where *bfpA* probe was considered as a method of diagnosis in swine diarrhoea. In that study, none of the 32 *E. coli* O45 isolates reacted with *bfpA* probe, but there were 25 and 11 positive strains for *eaeA* and EAF probe

respectively [Zhu *et al.* 1994]. A series of further studies must be undertaken to determine the efficacy and exact significance of this probe in EPEC diagnosis.

5.3.8. Role of serogrouping at present

Unfortunately, it was not possible to categorise EAF+ EPEC strains isolated from this study by serogrouping. It would be interesting to know the serogroups of these strains and to correlate the results of serogrouping with that of the probes.

All except a few recent studies related to EPEC have been carried out by serogrouping. It is becoming more complicated to interpretate and to correlate the results of serogrouping with those of the newly identified gene probes, and until now most of the investigators have to depend on serogrouping for the epidemiology of EPEC, as gene probing are not available in most of the laboratories. It is much more expensive to set up a molecular biology laboratory with all the necessary facilities.

5.3.9. EPEC in Hong Kong

Prevalence of EPEC in Hong Kong has been demonstrated previously by serogrouping and HEp-2 cell assay. The study, without control subjects, was based on serogrouping and showed 12.9% of EPEC in 442 cases [Lam *et al.* 1989]. However, it should be noted that serogrouping may overestimate EPEC [Morris *et al.* 1992]. The other Hong Kong study [Yam *et al.* 1987] identified 2.9% EPEC by serogrouping, of which only 2 patients were positive with HEp-2 cell assay. EPEC strains were not identified from any of 100 controls. Our results correlate well with those of Yam *et al* performed in 1987.

EPEC are commonly associated with low socio-economic status, poor nutritional status and usually transmitted through faeco-oral route in a poor hygienic environment [Regua *et al.* 1993; Abbar *et al.* 1991]. Although Hong Kong is still classified as a developing country, it has attained a very fast socio-economic development. In terms of various health and socioeconomic indicator Hong Kong should be classified as a developed country. As our data shows, the people of Hong Kong are very conscious about drinking water and they are being provided with a safe drinking water, so there is very little possibility of infection by polluted water. Overall child heath in this country is comparable to, or exceeds that, in many so called developed countries. In addition, EPEC is not a major cause of diarrhoea among the surrounding countries of Hong Kong [Kain *et*

al. 1991; Kim *et al.* 1989; Lim *et al.* 1992]. It can therefore be concluded that EPEC is unlikely to be a major cause of diarrhoea in this locality.

5.4. PREVALENCE OF OTHER CATEGORIES OF *E. COLI*

E. coli colonies from 253 cases and 177 controls were also investigated for Diffusely adherent (DAEC), Enteroaggregative (EAggEC) and Enterotoxigenic *E. coli* (ETEC). None of the probes were positive in cases significantly more than controls, thereby showing no association with diarrhoea for these pathogens. As mentioned before, no tests were performed for the identification of enteroinvasive *E. coli* and enterohaemorrhagic *E. coli.*

ETEC are a major cause of gastroenteriris in most developing countries [Guerrant *et al.* 1983], and also responsible for diarrhoea in travellers to the tropics [Mattila *et al.* 1992]. But the epidemiology is quite different in Hong Kong. In a study 2.5% of cases were infected by *E. coli* strains belong to ETEC serogroups [Lam *et al.* 1989], and enterotoxin producing ETEC were detected from 0.9% of children in Hong Kong in another study [Yam *et al.* 1987]. This helps us to conclude that ETEC are not associated with diarrhoea in Hong Kong, and this is probably due to better socio-economic status and environmental hygiene.

EAggEC has been implicated as a cause of persistent diarrhoea in children from different places [Bhan *et al.* 1989; Cravioto *et al.* 1991]. Persistent diarrhoea is very rarely found in Hong Kong and there was no significant association of diarrhoea with EAggEC in the present study.

Diffusely adherent *E. coli* (DAEC) is an unestablished category of *E. coli*, and its pathogenicity remains controversial [Thielman, 1994]. Whereas studies in Mexican [Mathewson *et al.* 1987; Giron *et al.* 1991a] and Bangladeshi [Baqui *et al.* 1992] children determined associations of DAEC with both acute and persistent diarrhoea. Our study demonstrated that DAEC is also not an important cause of childhood diarrhoea in Hong Kong.

5.5. COMMON AETIOLOGY OF DIARRHOEA IN HONG KONG

All stool specimens were cultured for bacteria and also tested with ELISA for rotavirus (in cases only). Microscopy was performed for parasites. The results were shown in the table-4.20. One or more aetiologic pathogen(s) were isolated in over 60% of cases. These results indicate the predominant pathogens which cause childhood diarrhoea in Hong Kong.

5.5.1. Rotavirus as the most common cause

Rotavirus was the most commonly detected pathogen. This is similar to industrialised countries [Kotloff *et al.* 1988] and some developing countries [Georges *et al.* 1984; Echeverria *et al.* 1989; Kim *et al.* 1989; Moyenuddin *et al.* 1987; Khalil *et al.* 1993]. Our results are consistent with other studies performed in Hong Kong before 1990 [Tam *et al.* 1986; Lam *et al.* 1989; Ling and Cheng, 1993]. Rotavirus demonstrated the most marked seasonal changes, peaking during the winter (December-February) and also in a higher rate in March and April, when the weather was relatively drier and cooler. Faeco-oral route has been described as the major route of transmission of rotavirus infection by World Health Organisation in 1979. In Hong Kong, respiratory tract infection is one of the most common causes for paediatric hospitalisation and rotaviral infection can also be spread as droplet infection. It has been documented that rotavirus was responsible for nosocomial infection in 20% cases of all rotavirus infection among the children admitted with acute gastroenteritis [Lam *et al.* 1989]. In the present study, rotavirus was not evaluated in the controls. But as asymptomatic rotavirus infection has been isolated in some places including Beijing, China [Kain *et al.* 1991; Barron-Romero *et al.* 1985; Champsaur *et al.* 1984], it would be useful to know the rotavirus prevalence rate in asymptomatic children in Hong Kong as well.

5.5.2. Non-typhoid *Salmonella* as the major bacterial pathogen

Contribution of non-typhoid *Salmonella* in children with diarrhoea in Hong Kong has been documented in previous studies [Ling *et al.* 1987; Ling and Cheng, 1993; Lam *et al.* 1989; Yam *et al.* 1987], most of which were conducted before 1985. The result of the present study confirms the result of other studies. *Salmonella* has also been identified as one of the

common causes of childhood diarrhoea in some of the surrounding countries, i.e. in Singapore [Lim *et al.* 1992], Thailand [Echeverria *et al.* 1989] and Beijing [Kain *et al.* 1989]. In contrast to these results, two recent studies, one from Israel [Lerman *et al.* 1994] and the other one from Bangladesh [Hoque *et al.* 1994] isolated only a small number of *Salmonella* from cases of childhood gastroenteritis. There is a good hygienic condition and safe water supply with better socio-economic status in Hong Kong, but still gastroenteritis caused by *Salmonella* is a significant problem in this city. It has been demonstrated that *Salmonella* commonly isolated from pigs for human consumption in Hong Kong were very similar to those found in humans [Chau *et al.* 1977]. The route of infection of *Salmonella* is usually from contaminated meat but spread to other foods may occur during their preparation and the epidemic of *Salmonellosis* in UK in 1985 has been attributed largely to poultry and hens' eggs [Cooke, 1990]. Here it also may be due to consumption of contaminated poultry and eggs, as Hong Kong depends heavily on imported food from different parts of the world. Thorough cooking of all food likely to be affected and careful handling of animal carcasses to reduce spread of the organism, are suggested to be an acceptable approach for the prevention of *Salmonella* food-poisoning. The food habit of the local people of taking relatively under-cooked food may also be a reason of its high incidence in this population.

The rate of non-typhoid *Salmonella* gastroenteritis particularly under 4 months of age in this territory may be contributed to lack of breast-feeding and early introduction of solids. The weaning food has a high possibility to be contaminated during preparation. Potential interventions against acute diarrhoea by education of parents and other primary care-givers on hygienic practices can prevent infectious diseases among the young children. Encouragement of lactating mothers to exclusively breast-feed their babies for at least initial 4 months [Lerman *et al.* 1994], would reduce the transmission of *Salmonella* through food preparation. It will also provide sufficient immunity to prevent easy infection and may play an important role in lowering the incidence of diarrhoeal diseases.

5.5.3. *Campylobacter* and *Shigella* as cause of diarrhoea

In this study, *Campylobacter* has been shown to be the third most common cause of diarrhoea in Hong Kong children. This is similar to previous studies [Lam *et al.* 1989; Yam *et al.* 1987]. In some developing countries like Bangladesh, it was isolated as the

commonest cause (26%) of childhood diarrhoea in a clinic based study and predominantly infected children under 2 years [Hoque *et al.* 1994]. It usually behaves as an waterborne, milk-borne and zoonotic disease [Murga *et al.* 1995]. Like *Salmonella*, it may also be transmitted from infected poultry and contact with pets. Although it does not multiply in food, there have been outbreaks attributable to contaminated milk and water supplies [Cooke, 1990]. Furthermore, it has been demonstrated by a multi-centre case-control study in London that occupational exposure to raw meat, pets with diarrhoea and untreated water are still important risk factors for *Campylobacter* infection [Adak *et al.* 1995]. The exact factors for a continued *Campylobacter* diarrhoea in Hong Kong are not studied.

Shigellosis is not a major health problem for Hong Kong children, although it is an important cause of severe diarrhoea in children from the developing world [Ebrahim, 1991]. In Hong Kong, this has been achieved due to overall development of socio-ecnomic status and hygienic conditions. The age distribution and clinical findings of these cases were as expected, the only notable thing was that all *Shigellosis* were caused by *Shigella flexneri*.

5.5.4. Role of parasites in childhood diarrhoea in Hong Kong

The absence of parasites in this study reflects the rapid urbanisation of the territory with improving sanitary practices. This findings do not correlate with the study [Kam, 1994] performed in 1992 (total 8.6% positive) but it concurs with the rapid decline of parasitic infestation rate from 28.7% of positive cases between 1979 to 1981 [Duchastel, 1983] to 8.6% in 1992. The failure to isolate *Giardia* and other parasites in this study also may be due to the technical problems of our sample collection and delay in microscopy. The samples were not examined immediately after collection and in some cases, it was processed after 48 hours.

5.6. CONTROL MEASURES FOR DIARRHOEAL DISEASES

5.6.1. Prevention of diarrhoea through improved nutrition

Control of diarrhoeal diseases (CDD) programme of the World Health Organisation (WHO) has got a strategy to prevent diarrhoea through improvement of nutrition. It could be achieved in young infants by exclusive breast feeding for initial 4-6 months and good weaning practices. Breast feeding should be started as early as possible. Colostrum contains very high number of immunoactive white blood cells [Ogra and Ogra, 1978] and other anti-infective factors, the concentration of which decrease rapidly in the 48-72 hours after birth. Furthermore, early breast feeding provides some protection against diarrhoea later on [Gunnlaugsson *et al.* 1995]. There is every chance of contamination of weaning food during preparation. It has been reviewed by Motarjemi *et al* in 1993 that weaning food prepared under unhygienic conditions are frequently heavily contaminated with pathogens and thus are a major factor in the diarrhoeal diseases and associated malnutrition. Suggestion for the education of mothers in food safety principles, particularly weaning food has also been made so that mothers should prepare and store food properly. It has also been demonstrated that improvements on weaning practices can result in better nutritional status of the infants, reduce food contamination [Walsh *et al.* 1979] and thereby prevent diarrhoea. Enteropathogens, in particular water-borne bacteria and parasites, cause diarrhoea by contamination of food in poor hygienic conditions. However, it is possible to reduce diarrhoeal morbidity by ensuring safe water supply and also through promotion of personal, domestic as well as environmental hygiene.

In the management of acute diarrhoea, it is essential to prevent nutritional damage. This is best done by continuing to give nutritious food during diarrhoea and giving extra food for 2 weeks after diarrhoea stops. In breast feeding infants, it should be continued like before, because withdrawal of breast feeding during diarrhoea has been identified as one of the risk factors for development of life-threatening dehydration in young children with acute diarrhoea [Bhattacharya *et al.* 1995]. Diarrhoea leads to malnutrition by loss of appetite, food withdrawal during diarrhoea and reduced absorption of nutrients. Malnutrition increases the duration and severity of diarrhoea. It had been shown by Black *et al* in 1984 that among children <24 months in Bangladesh, diarrhoea duration increases

as nutritional status decreases. Mortality due to diarrhoea was 3 to 7 times higher in infants with malnutrition [Chen *et al.* 1980].

5.6.2. Fluid supplementation in diarrhoea

Fluid supplementation during illness is of prime importance particularly in those diseases where the patient loses excessive fluid. Oral rehydration solution (ORS) has been proven to be very useful for rehydration of diarrhoea patients for more than 25 years. WHO recommended it as the key to the management of diarrhoea. ORS can be used alone to rehydrate 95% or more patients with some dehydration. Patients with severe dehydration require rehydration with IV fluids initially, but ORS should be used after the initial deficit has been corrected. In this study, only 15.5% of the diarrhoea patients had ORS at home. Although only a quarter of the total cases were dehydrated, ORS could be still useful before any signs of dehydration appear, when the patient has increased fluid loss from body. Early home therapy to prevent dehydration due to diarrhoea is an important educational message. When oral fluid therapy is given to children at home early during diarrhoeal illness to prevent dehydration, it can substantially decrease the number of visits to hospital and overall diarrhoeal disease mortality and morbidity rates as well. Home fluids can be used to continue the treatment of children at home after they have been discharged from the hospital. A recommended home fluid can be given, which may be a food-based fluid such as cereal, soup or rice-water. The food based fluids should be as thick as possible while still being drinkable. Now a days, rice based-ORS has been proved as a better alternative or even superior to standard glucose-ORS in the management of diarrhoeal diseases in children through a series of clinical studies [Prasad, 1993; Islam, A. *et al.* 1994; Maulen-Radovan *et al.* 1994]. It could be used through in-house preparation and as a cost effective measure in treating diarrhoea patients [Islam, MA. *et al.* 1994].

5.6.3. Strategies to control diarrhoea

In Hong Kong, future diarrhoeal disease preventive strategies should include protection of environment, ensurance of safe water supply, maintenance of high standard of environmental hygiene and sanitation and promotion of the practice of good personal and food hygiene. Emphasis should be given in developing sewage treatment and disposal programme to reduce the pollution of coastal waters.

5.6.4. Health education

Community participation and health education can play a significant role in health promotion and disease prevention, as transmission of gastrointestinal communicable diseases is often due to inadequate personal and food hygiene and unsatisfactory sanitation.

In this study, the mean duration of hospital stay in the diarrhoea group was 3 days. This is a quite long time for the mild diarrhoeal cases. It has been clearly demonstrated that majority of diarrhoea cases were mild in nature. Furthermore, only a quarter of cases had some dehydration and none had severe dehydration. Over 95% of cases with some dehydration could be managed well at home by oral rehydration therapy. This needs health education for the parents to teach them how to treat diarrhoea at home by a) giving the child increased fluids, b) continuing to feed and c) recognising the signs indicating that they should bring their child to a health worker. The promotion of home management certainly can reduce financial costs of medical care and also can save working hours for both hospital staff and the parents. It will also decrease the possibility of nosocomial infection in hospital for the young children. Exclusive breast feeding for the first 4-6 months of life and partially for at least 1 year, is another important message from WHO, which can successfully prevent diarrhoeal illness in the infants [Popkin *et al.* 1990].

Community participation in health education and publicity not only promotes better understanding and awareness of the diseases but also encourages the adoption of appropriate measures in disease prevention.

In our experience, persistent diarrhoea is very rare in Hong Kong, unless it is complicated by other gastrointestinal diseases. The relatively mild clinical features in these diseases could be due to adequate treatment facilities and good nutritional status of the children, which provides a better immune system to prevent further deterioration. However, the success achieved in the control of diarrhoeal diseases in Hong Kong might be largely due to public health efforts, effective medical care together with improved living standard and hygiene practices.

Chapter-6

CONCLUSION

Results obtained from the present study showed that diarrhoea is a common disease in Hong Kong, particularly for the young children. Although the mortality due to diarrhoea in Hong Kong has been reduced to a level comparable to those of highly developed countries, morbidity of the disease remains an important problem.

Rotavirus, which is seen particularly in the winter, and *Salmonellosis*, an endemic cause of diarrhoea, were the most important pathogens isolated. Different categories of *E. coli* including EPEC and parasites were not important causes of diarrhoea. Health education of parents and other primary care givers on hygienic practices can successfully prevent transmission of diarrhoeal pathogens to the infants through weaning foods. This might be particularly important to the prevention of *Salmonellosis*. Proper management of diarrhoea at home through replacement of fluid and continuing to feed may reduce diarrhoeal morbidity as well as the rate of hospital admission. Further improvement of food hygiene and environmental sanitation, and above all, encouragement of mothers for exclusive breast feeding their babies may further reduce diarrhoeal morbidity in Hong Kong.

Chapter-7

APPENDIX

7.1. QUESTIONNAIRE

The Prevalence of Enteropathogenic *Escherichia Coli* (EPEC) and Other Pathogens in Hospitalised Children with Diarrhoea in Hong Kong

Date: **Study/Control no:** **Name:**

Sex: M/F **Age:** months **HN** **HKID**

Area of abode in Hong Kong: CHIna/East N.T./ West N.T/ HK Island

Socio-econ status: 1,2,3,4,5 / Father's employment

Educational status: School/ Kindergarten/ Neither

Mother's education:(up to age).....yrs.

Ethnic origin: CHInese (CANtonese/OTHer) VIetnamese (Nth/Sth)/ CAUcasian

Diarrhoea-.....hours duration. **Vomiting-**.....hours duration

.....stools per day .....Vomit per day

watery(Y/N) mucoid(Y/N) bloody(Y/N) bile(Y/N) blood(Y/N)

Main Illness Intake: Formula/ Breast milk/ Cow's milk/ Normal diet/ Other

Supplementary Intake: Water/ ORS/ Fruit-juice/ Other

Urine output: Normal/ Reduced/ None for 12 or >12 hours

Other features: Fever/ Anorexia/ Abdominal pain/ Rash/ Arthritis/ Headache

Early feeding History: Breast milk. Y/ N What age stopped? .....months

Bottle (unboiled/boiled, tap/bottled water) Weaned at .....months

Drug history: Antibiotics in past 2 weeks - Y/N name?

Antidiarrhoeal agents this illness- Y/N name? Other drugs-

Family History: Diarrhoea? Fa/Mo/Sib/Amah/Other **Fever?** Fa/Mo/Sib/Amah/Other

Social History: No.in household- **H/O recent** (last 2 weeks) **travelling-** Y/N

Diarrhoea contact? Y/N - Play-mates/ Relatives/ School friends/ Others

Source of drinking water - Tap(boiled/unboiled) / Bottle

Examination Findings

General Exam:

Weight:.....kg (%) Height:..... cm (%) BMI=W/H^2 (W in Kg,H in m)

Temp.....°C Pulse- /min Resp rate-...../min

Level of Dehydration:

	<5%	**5-9%**	**10%+**
Sensorium	normal/alert/restless	lethargic	limp/unresponsive
Pulse	normal volume	weak	very difficult to feel
Bp	normal	normal	Hypotension <80mmHg Syst
Cap. perfus^n	normal	reduced	pale/cyanosed peripheries
		(cap return 2-3secs)	*(cap return >3secs)*
Muc Memb	moist	dry	dry
Skin turgor	normal	reduced	very reduced
	(immediate retraction)	*(retraction<2s)*	*(retraction>2s)*
Eyes	normal	sunken(slight)	sunken(gross)
Ant Font	normal	sunken (slight)	sunken(gross)

ENT examination: Otitis (Y/N) Pharyngitis (Y/N)

Abdomen: Distension(Y/N) Tenderness(Y/N)

Bowel-sound- present/absent

Perineum- Healthy/ Mild erosion/ Excoriation

Other Major Diagnoses:

Laboratory Findings

1.**Microscopy-**Ova/Cyst of...../ Neg/ Not done 2. **Culture-** Neg/ Pos (name oforganism)

3. **E. coli-** Neg/ Pos (name of category) 4. **RV Elisa**: Neg/ Pos/ Not done

Outcome

Total Duration of Diarrhoea .....hours

(from 1st to last loose stool)

Duration of Hosp Admission.....hours

I/V infusion- N/Y .....ml

Drugs used in hospital-

Other- ICU/ Death

7.2. LABORATORY METHODS

All samples were examined under microscope for ova and cysts of enteric parasites. The specimens were also cultured routinely for the identification of the following pathogens: *Salmonella*, *Campylobacter*, *Shigella*, and *Aeromonas*. Thiosulphate Citrate Bile Sucrose (TCBS) agar medium (1/2 plate) was used for *Vibrio cholerae* on all stool samples. In addition, an enrichment culture was performed in the special circumstances when the stool specimen was watery or it was received from a clinically suspected case of cholera. The detailed methods for culture of the above mentioned pathogens, preparation of slides and methods for microscopy of the ova and cysts of different parasites are all described below.

7.2.1. Routine culture of stool specimens for bacteria

A. Routine culture media and incubation conditions in PWH Microbiology Laboratory

Routinely, stool samples were inoculated in the following media and incubated at conditions stated:

1) Desoxycholate Citrate agar (DC) plate, 37^0C aerobically for 18 to 24 hours;

2) Selenite-F (SF) enrichment broth, 37^0C aerobically for 18 to 24 hours;

3) Thiosulphate Citrate Bile Sucrose agar (TCBS) plate, 37^0C aerobically for 18 to 24 hours;

4) Skirrow's agar plate, incubated under microaerophilic conditions at 42^0C for 48 hours.

Microaerophilic conditions were obtained by using an anaerobic jar without a catalyst. The jar was evacuated to 500 mm of Hg and replaced with a gas mixture (10% H_2, 10% CO_2 and 80% N_2). A standard *Campylobacter* strain, NCTC 11351, was used as control for the Skirrow's medium, and was subcultured everyday.

B. Culture examination and identification

After incubation for a particular period as mentioned above, each culture plate was examined and colonies were identified in the following ways:

1) Desoxycholate Citrate agar (DC)

a) At first the media was examined for non-lactose fermenting and late-lactose fermenting colonies.

b) Two (if predominant growth of same colonial morphology) or more (if colonies were of different morphology) colonies were picked from the DC plate using a straight wire and were inoculated into each of the following tubes:

- Triple Sugar Iron (TSI) slant (contains glucose, lactose, sucrose and iron);
- urea peptone broth;
- tryptone water (for detection of indole production);
- dulcitol; and
- mannitol.

Then they were streaked on a MacConkey agar plate to check for purity of growth.

c) These tubes were incubated at 37^0C for 18-24 hours, and

d) Reactions in TSI slant were interpreted according to the following table:

Table- 7.1 Reactions in TSI slant

Butt	Slope	H_2S (blackening of medium)	Interpretation
Acid	Alkaline	Yes	*Salmonella* sp.
Acid	Alkaline	No	Shigella sp, Aeromonas.
Acid	Acid	Yes	Some *Citrobacter* strains.
Acid	Acid	No	*E. coli*, *Klebsiella*, *Enterobacter* sp, *Aeromonas.*
Alkaline	Alkaline	No	Non-fermenting bacteria

e) If reaction was suggestive of enteric pathogens, then oxidase test was performed and readings were noted from the following biochemical reactions:

Table- 7.2 Biochemical reactions for bacteria

Test	Salmonella	Shigella	Aeromonas
Oxidase	-	-	+
Urease	-	-	-
Indole	-	-/+	-/+
Dulcitol	-	-/+	-
Mannitol	-	dys -/+	+

f) The findings were confirmed by API20E or Microbact 24 E.

g) Whenever the growth was suspected of *Salmonella* or *Shigella*, serotyping was also performed by slide agglutination. It has been described in details later in this section.

h) If colonies on primary plates were suspected to be *Salmonella* or *Shigella*, then serotyping and biochemical tests were performed as described earlier in this section.

2) Selenite-F enrichment broth

At first it was subcultured into a DC plate and then the DC plates were examined on the next day as described earlier (methods for DC plate).

3) Thiosulphate citrate bile sucrose agar (TCBS)

a) The plate was examined for well-formed yellow colonies on primary TCBS plate or on TCBS plate subcultured from Alkaline peptone water (APW) and bluish, transparent colonies on primary Monsur's (MON) plate or on MON subcultured from APW. The methods of identification in details are described later in the current chapter.

b) Well-formed green colonies were looked for and then they were subcultured onto blood agar (BA). Oxidase test was performed on the next day. If the colony was oxidase positive, biochemical identification was performed by API20E (emulsifying colonies in normal saline and using as inoculum).

4) Skirrow's agar

a) The medium was examined for a fine transparent film of growth resembling swarming *Proteus* (some *Campylobacters* form discrete colonies) or 'tear-drop colonies'. A Gram smear was prepared from the primary inoculum (counterstained with 0.2% carbol fuchsin) and was examined for small spiral or S-shaped Gram-negative bacilli. Oxidase test was also performed to confirm the identification.

7.2.2. Serotyping of Salmonella, Shigella and Vibrio cholerae

1. Method for slide agglutination test

While the test was being performed, colonies growing on a selective medium was never used, as erroneous results might be obtained.

a) A small amount of growth from a pure culture was picked on nutrient or blood agar (or from TSI slant) and, using a platinum loop, it was emulsified in saline to give a smooth suspension on a clean glass slide.

Another such suspension on the same slide was prepared to act as a negative control and a standard *Salmonella* strain was used as positive control.

b) A small drop of antiserum was added on the slide just above the emulsion and the two drops were brought into contact using the platinum loop. It was mixed by rocking the slide gently backwards and forwards.

c) Then it was looked for visible clumping (agglutination) within 60 sec of mixing between the suspension and antiserum. Each time agglutination was compared with the controls.

d) The slide was finally discarded into a discard jar.

2. *Salmonella* serotyping

1. Initial screening

a) Suspected organisms by slide agglutination (described above) was tested against polyvalent O, polyvalent H (specific and non-specific phase), polyvalent H (non-specific phase), and Vi antisera.

b) An organism which did not agglutinate with polyvalent-O or Vi antisera was unlikely to be a *Salmonella*.

2. Determination of serogroup (O antigen)

a) If organism agglutinated in polyvalent-O antiserum, it was then retested with monovalent sera to determine the serogroup.

b) If polyvalent-O agglutination was negative but Vi agglutination was positive, the Vi antigen was removed and the strain was retested, as follows:

1) Organism was emulsified in 1 ml saline and was heated for 30 minutes at 100^0C;

2) After centrifuging at 12 000 g for 5 min, the supernatant was discarded and was resuspended pellet in 0.5 ml saline;

3) This suspension was used to repeat agglutination test with polyvalent-O antiserum.

3. Determination of serotype (H antigen)

a) If organism agglutinated in polyvalent-H (specific and non-specific) antiserum but not in polyvalent-H non-specific antiserum, then it was in the specific phase.

b) It was tested with individual H antisera of the specific phase.

c) If organism agglutinated strongly in polyvalent-H non-specific antiserum, it was in the non-specific phase. Then it was tested with individual H antisera of the non-specific phase.

d) It was changed to the specific phase using the bridge plate method before testing individual H antisera of the specific phase was performed as follows:

1) Using sterile scalpel blade; a ditch 1 cm wide across a blood sugar plate was made.

2) The trough was bridged with a piece of sterile filter paper.

3) The test *Salmonella* was inoculated on one side of the filter paper with the culture and a drop of polyvalent-H non-specific phase antiserum was placed on the other side.

4) It was incubated for 18 hours at 37^0C.

5) Motile organisms migrated across the bridge, but those in the non-specific phase were immobilised by the antiserum. Then it was subcultured onto blood agar and was determined H antigen (specific phase) by slide agglutination.

6) If organism was found still in the non-specific phase, the process was repeated.

3. *Shigella* serotyping

Suspected organisms by slide agglutination against polyvalent antisera were tested. If organism agglutinated in one of the polyvalent-O antisera, then it was retested with monovalent antisera to determine the serotype.

4. *Vibrio cholerae* serotyping

Suspected colonies on Monsur's agar or blood agar by slide agglutination with antiserum was tested to *V cholerae* 01 and 0139.

Media used in stool culture

1. TCBS agar medium

Ingredients: a) TCBS agar ----------44 g

b) Distilled water -----500 ml

was mixed and dissolved by steaming (not autoclaved). Then it was cooled, poured plate immediately and stored at 4^0C.

2. DC agar medium

Ingredients: a) Lab.-Lemco --------- 100g

b) Peptone ---------- 100g

c) Agar ---------- 200g

d) Water ---------- 20 litre

These were mixed and dissolved by steaming in autoclave for 1 hour. It was made up to 20 litre, pH was adjusted to 7.4 with Lovibord. 400 ml was dispensed into each bottle and sterilised by autoclaving under 15 lb. for 15 minutes.

For use, DC A solution and B solution, 15 ml from each were added with 2% neutral red solution (0.5 ml). These were mixed with 10 ml of 40% Lactose solution, mixed and poured onto plate.

3. Selenite F medium

Ingredients: a) Sodium Hydrogen Selenite (Oxoid) ---- 20g

b) Lactose ---------- 20g

c) NaH_2PO_4 (Anhydrous) ---------- 19.7g

d) $NaH_2PO_4.H_2O$ ---------- 5.3g

e) Peptone ---------- 25g

f) Distilled water ---------- 5 litre.

These were mixed and dissolved, pH was adjusted to 7.1. Then 2 ml of concentrated HCl was added. 12 ml was dispensed to U- bottle. Finally it was sterilised by steaming at 100^0C for 16 minutes and took out promptly.

4. Skirrow's medium or Campylobacter Lysed Blood Agar (TVP) medium

Ingredients: a) Nutrient agar base ---------- 400ml

b) Lysed horse blood ---------- 20ml

c) Reconstituted skirrow (Oxoid SR69) - 1.6ml

(Camp. selective supplements)

d) 10% FeSO4. 7H2O (Ferrous sulphate)- 1ml

e) 10% Sodium metabisulphate ---------- 1ml

f) 10% Sodium pyruvate ---------- 1ml

At first the Nutrient agar base was melted and then b) to f) were added. It was mixed and poured onto plate.

7.2.3. Microscopic examination of ova and cysts

Direct wet mount (for trophozoite only) was not applied as all the stool samples were not possible to process within two hours of collection. The samples were examined after concentration and the method used is known as 'Formalin-ether concentration method of Ritchie', which has been described below in details.

a) Sample from different parts of stool, an amount of about 1.5 cm in diameter was taken and mixed with 10 ml formal saline.

b) It was strained through gauze into a 15 ml centrifuge tube.

c) Then 3 ml ether was added to the centrifuge tube.

d) It was shaken vigorously.

e) Centrifugation was done for 2000 rpm for 3 minutes.

f) Supernatant was decanted.

g) Residue was mixed with a vortex mixer.

h) Then a portion of the residue was examined under microscope and the size of ova or parasites identified was recorded. In case of difficulties in identifying ova or cysts, a drop of iodine solution was added and re-examined.

7.2.4. Laboratory diagnosis of rotavirus

Rotaviruses are non-enveloped RNA viruses consisting of a spherical inner core and two outer capsid shells. At least six serogroups(A-F) within the genus Rotavirus have been identified.

The laboratory diagnosis of rotavirus infection plays an important role in patient management and enables effective management and control of outbreaks. At present human serotypes of rotavirus do not grow readily in cell culture systems, hence they are difficult to isolate from clinical specimens. Therefore, the laboratory diagnosis of rotavirus can be performed using electron microscopy or radioimmunoassay to detect the virus or viral antigens, or polyacrylamide gel electrophoresis to detect RNA of the rotavirus genome. These procedures are technically demanding and require specialised equipment which limit their application.

More recently enzyme immunoassays, using specific monoclonal or polyclonal antibodies, have been described for the direct detection of rotavirus in clinical specimens. These tests offer a rapid, sensitive and specific method for the detection of rotavirus in faecal specimens.

DAKO IDEIA™ Rotavirus test was used for the detection of rotaviruses in faecal specimens of the cases. The IDEIA™ Rotavirus test is a qualitative enzyme immunoassay for the detection of rotavirus (Group A) in human faecal samples, which can be read visually or photometrically, as an aid in the diagnosis of acute gastroenteritis caused by Group A rotavirus. The procedure was followed as it was described in the IDEIA™ Rotavirus test kit information booklet.

Principle of the test:

The IDEIA™ Rotavirus test utilises a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect group specific antigen present in Group A rotaviruses. Break-apart microwells are coated with a rotavirus specific polyclonal antibody. Faecal suspension or control sample was added to the microwell and incubated simultaneously with a rotavirus specific polyclonal antibody conjugated to horseradish peroxidase. Rotavirus antigen present in the samples was captured between antibody on the solid-phase and the enzyme conjugated antibody. After 60 minutes incubation at room temperature, the microwells were washed with working strength washing buffer to remove

excess specimen and any unbound enzyme labelled antibody. A chromogen was added to the wells and incubated for 10 minutes at room temperature. The presence of specifically bound enzyme labelled antibody in the wells results in a colour change which was stopped by the addition of acid. Colour intensity significantly above background levels was indicative of the presence of rotavirus antigen in the specimen or control.

Specimen collection:

Faecal specimens for direct testing were collected into containers that did not contain media, preservatives, animal sera, metal ions, oxidising agents or detergents, as all of these additives might interfere with IDEIA™ rotavirus test.

Preparation of faecal specimens:

1 ml of sample diluent was added to a suitable labelled container and was used to prepare a 10% suspension or dilution of faecal specimen by addition of approximately 0.1 gm of solid faeces (small pea-sized portion) or approximately 100 µl of liquid faeces. It was mixed thoroughly and left to be settled for 10 minutes prior to testing.

Preparation of controls:

Negative control: 1 ml of sample diluent was added to a vial identical to those used for sample dilution.

Positive control: The positive control was ready to use within the kit. It was mixed gently before use.

Procedure:

1. 2 drops (100 µl) of negative control were added to the first well.
2. 2 drops (100 µl) of positive control were added to the second well.
3. 2 drops (100 µl) of each diluted supernatant of faecal specimens were added to the respective wells.
4. 2 drops (100 µl) of conjugate (rotavirus specific rabbit polyclonal antibody conjugated to horseradish peroxidase in a buffered protein solution containing antimicrobial agent and blue dye) was added to each well and was mixed gently.
5. It was covered and incubated at room temperature for 1 hour in a moist chamber.
6. Then it was washed 6 times using washing buffer concentrate (tris buffered solution containing antimicrobial agent and detergent).

7. 1 drop (50 µl) of substrate part A (substrate buffer containing tetramethyl-benzidine [TMB] and antimicrobial agent) and 1 drop (50 µl) of substrate part B (substrate buffer containing hydrogen peroxide and antimicrobial agent) were added to each well and mixed.

8. It was again covered and incubated at room temperature for 10 minutes in a moist chamber.

9. 1 drop (50 µl) of stopping solution (0.46 mol/L sulphuric acid) was added to each well and thoroughly mixed.

10. The absorbency was read using a suitable spectrophotometer set at 450 nm within 30 minutes after addition of the stopping solution. It was ensured that the bottoms of the wells were clean before reading and there was no foreign matter in the wells. The reader was blank on air (i.e. with no plate on the carriage) before the plate was scanned. Alternatively the spectrophotometer allows for the use of a reference wavelength (at 620 to 650 nm), dual wavelength reading should be performed as this eliminates any potential interference caused by aberrations, such as dirt or marks, on the optical surface of the wells.

Interpretation of results:

In photometric determination, the negative control value or mean of the negative control values, should be less than 0.150 absorbency units and the positive control must have a value greater than 0.500 absorbency units. The cut-off value was calculated by adding 0.100 absorbency unit to the negative control value.

Any clinical specimen with an absorbency value greater than the cut-off was considered as positive and that of less than the cut-off value was considered as negative. A result within 0.100 absorbency unit of the cut-off value was considered equivocal. Such results were interpreted in conjunction with patient related clinical/epidemiological information. Alternatively the specimen should be retested or the patient resampled.

The testing of meconium samples, which are from the early days of a neonate, are not been validated with this test. So, if such a sample was positive by this test, the result was confirmed by the Latex agglutination card test with Slidex Rota-Kit 2. It contains monoclonal antibody sensitised latex reagent for detection of rotavirus in faeces.

7.3. INVESTIGATION REQUISITION FORM

The ordinary investigation requisition form was made special by a rubber stamp mentioning EPEC study for easy identification. A sample is shown below.

Lab. No. №1187390

PRINCE OF WALES HOSPITAL
MICROBIOLOGY

Tel.: 636 2308

← Please tear off here ↓

H.K.I.D.

HOSP/OPD No.

Surname

Other Name

Sex/Age ...../.....

Date of Birth D M Y

Ward/Bed ...../..... or ☐ OPD

Doctor's Code

Unit Head's Code

Pager/Ext No.

M.O. Signature

Unit (mark a 'X')

OA & E	A & E
BURNS	Burns
CCU	CCU
ENDO	Endoscopy
GYNAE	Gynae
ITU	ITU
MED	Medical
NNU	Neonate
OBS	Obstetric
RTON	Oncology
ORTH	Orthopaedic
PAED	Paediatric
PSURG	Paed-Surg
PSY	Psychiatric
RT	RT
OSC	Staff Clinic
SURG	Surgery

Other (Specify)

Date & Time Collected

Date & Time Received

☐ URGENT

Clinical Diagnosis/History:

EPEC STUDY NO. ___
STOOL SAMPLE NO. 1

Present Antibiotic Therapy:

NATURE OF SPECIMEN *(Please tick ONE only)*

- BI Bile
- BC Blood culture
- BCT Blood (clotted)
- MA Bone marrow asp.
- CSF CSF
- CIV Catheter I.V.
- PLA Pleural asp.
- ASC Ascitic fluid
- PERI Peritoneal asp.
- PDF Peri-dialysate
- CAPD CAPD fluid
- SP Sputum
- TA Tracheal asp.
- ETT Endotracheal tube
- BA Bronchial asp.
- ☑ Stool
- RR Rectal swab
- UR Urethral swab
- JT Joint asp. Site
- TIS Tissue (Specify)

SWABS

- EA Ear [R] [L]
- EY Eye [R] [L]
- CS Endocervical
- HVS High vaginal
- VVS Vulval
- NS Nasal
- MS Oral
- TS Throat
- UMB Umbilical
- SK Skin Site
- SS Skin scrape Site
- SW Superficial wound Site
- DW Deep wound Site
- PS Pus Site

URINE

- BU Bag urine
- CU Catheterized
- EMU Early morning
- MSU Mid-stream
- NU Nephrostomy [R] [L]
- SPU Suprapubic tap
- UU Ureter [R] [L]
- DIP Dipslide

- SRT Sterility test Type
- ENV Environmental Type
- PHRM Pharmacy Type
- ☐ Other (Specify)

INVESTIGATION

- ☑ Routine Microscopy +Culture

MICROSCOPY

- CC Cell count
- RBC Red blood cell
- CAS Casts
- CNEO Cryptococcus
- FG Fungus
- PAR Parasites
- AMOE Amoeba
- SPI Spirochetes
- CHY Chyle

CULTURE

- GCC Gonococcal
- FGC Fungal

SEROLOGY

- ASO ASO titre
- BR Brucellosis
- PRT Pregnancy test
- WF Rickettsial
- VD Syphilis
- WID Typhoid

Acid fast bacilli

☐ Smear ☐ C & ST
Previous Rx ☐ Y ☐ N
Present Rx ☐ Y ☐ N

- AA Antibiotic assay
  - ☐ Predose
  - ☐ Post dose

Drug to be assayed

Loading dose

Maintenance dose

Dosing Interval ..... hrs
Blood creatinine Level ..... umol/
Body weight ..... kg
Height ..... m. ..... cm

☐ Other (Specify)

HA 1884 (Rev. 5/92)

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GRADUATE SEMINARS & PUBLICATIONS

a. Graduate seminars

- Seminar-1. Cow's Milk Protein Intolerance as a cause of Diarrhoea.
- Seminar-2. Intestinal Permeability Testing by Lactulose and Mannitol.
- Seminar-3. The Prevalence of Enteropathogenic *Escherichia coli* with Diarrhoea in Children in Hong Kong.
- Seminar-4. Aetiology of Acute Diarrhoea in Hospitalised Children in Hong Kong.

b. Publications

1. D. Lau, R. Biswas, P.J. Lewindon, P.B. Sullivan. Cow's Milk Protein Intolerance as a cause of Chronic Diarrhoea in Hong Kong Children (Abstract). Journal of Gastroenterology and Hepatology, 1994, 9(6), A168.

2. R. Biswas, D.J. Lyon, E.A.S. Nelson, D. Lau, P.J. Lewindon. Aetiology of Acute Diarrhoea in Hospitalised Children in Hong Kong. Abstract presented in 21st International Congress of Pediatrics, Cairo, Egypt, September 10-15, 1995 and manuscript submitted for publication.

3. E. Chow, E. A. S. Nelson, R. Biswas, P.J. Lewindon. Management of Acute Diarrhoea in Hong Kong (Abstract). 21st International Congress of Pediatrics, Cairo, Egypt, September, 1995.

4. R. Biswas, E.A.S. Nelson, P.J. Lewindon, D.J. Lyon, P.B. Sullivan, P. Echeverria. Molecular Epidemiology of *Escherichia coli* Diarrhoea in Hong Kong Children (in preparation).